Université de Montréal

Régulation moléculaire et cellulaire de l'efflux de cholestérol par le transporteur ATP-binding cassette A1 (ABCA1)

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Thèse présentée à la Faculté des études supérieures
En vue de l'obtention du grade de
Philosophiæ Doctor (Ph.D)
En biochimie

Août 2004

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée

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Résumé

Les patients atteints de la maladie de Tangier présentent des niveaux de lipoprotéines de haute densité (HDL) anormalement bas, un facteur de risque pour le développement de maladies cardiovasculaires. *In vivo*, les HDL ont un effet athéroprotecteur important puisqu'elles effectuent le transport à rebours du cholestérol des tissus périphériques vers le foie. Or, la maladie de Tangier est causée par des mutations dans le gène du transporteur « ATP-binding cassette A1 » (ABCA1). Le modèle actuel veut que ce transporteur promeuve la lipidation de l'apolipoprotéine A-I (apoA-I), la composante protéique majeure des HDL, pour former des particules HDL naissantes discoïdales ayant une migration électrophorétique en position pré-β. Un défaut dans la lipidation de l'apoA-I par l'ABCA1 abolit la biogénèse des HDL. Aussi, nous avons voulu étudier la régulation transcriptionnelle de l'ABCA1, son interaction avec son ligand (l'apoA-I), les particules qui en résultent, et la conformation structurale requise du transporteur pour effectuer sa fonction. D'abord, en utilisant un inhibiteur de facteurs nucléaires spécifiques, il a été établi qu'une charge en cholestérol induit la transcription d'ABCA1 via la génération d'hydroxystérols. Ensuite, nous avons utilisé ce système inductible afin de déterminer la nature du contact entre l'apoA-I et l'ABCA1. Une étude de radioliaison avec prétraitement des membranes cellulaires aux phospholipases n'affecte pas la liaison, excluant une interaction indirecte médiée par des domaines lipidiques. Contrairement au modèle proposé plus haut, nous avons établi par gel bi-dimensionnel que la lipidation de l'apoA-I par l'ABCA1 génère des HDL naissantes migrant plutôt en position alpha. Finalement, par pontage moléculaire nous avons montré que la liaison de l'apoA-I requière l'organisation de l'ABCA1 en une structure oligomérique. Cette oligomérisation résulte en la formation de particules alpha composées de plusieurs molécules d'apoA-I. Ces observations réunies démontrent que 1) l'apoA-I s'associe à l'ABCA1 oligomérique dans une interaction protéine/protéine directe; 2) cette interaction résulte en la formation de HDL naissantes contenant plusieurs molécules d'apoA-I phospholipidées migrant en position alpha. En conclusion, les résultats présentés dans ce travail bouleversent complètement les modèles préalablement établis et proposent de nouveaux concepts et avenues de recherche sur l'homéostasie du cholestérol par les HDL.

Mots clés : cardiovasculaire, cholestérol, efflux, HDL, ABCA1, apolipoprotéine A-I

Abstract

Patients with Tangier disease have very low levels of high density lipoproteins (HDL), a known risk factor in the development of cardiovascular diseases. *In vivo*, HDL are atheroprotective as they promote reverse transport of cholesterol from peripheral cells back to the liver. The molecular defect for Tangier disease has recently been identified : mutations in the ATP-binding cassette transporter A1 (ABCA1). The current model suggests that ABCA1 promotes the lipidation of apolipoprotein A-I (apoA-I), the major protein component of HDL, to form pre-β-migrating, discoidal nascent HDL particles. A defect in the lipidation process of apoA-I by ABCA1 abolishes the generation of HDL. Thus, the aim of this work is to study the transcriptional regulation of ABCA1 and the interaction with the ligand apoA-I, to characterize the resulting particles and to define the functional structure of ABCA1. First, using an inhibitor of specific nuclear factors, we have demonstrated that cholesterol loading, through the conversion to hydroxysterols, induces ABCA1 transcription. Second, we used this inducible system to determine the nature of the interaction between apoA-I and ABCA1. Pretreatment of the plasma membrane with phospholipases did not alter the binding of ^{125}I-apoA-I to ABCA1, thus ruling out a possible indirect contact between the two molecules. Arguing against the aforementionned model, we found, by two-dimentional gel electrophoresis, that the lipidation of apoA-I by ABCA1 generates alpha-migrating, nascent HDL particles. Third, a molecular cross-linking experiment revealed that the binding of apoA-I requires the assembly of ABCA1 monomers into oligomeric structures. This oligomerization results in the formation of alpha-migrating particles containing many apoA-I molecules. Together these data suggest that apoA-I binds to an oligomeric ABCA1 in a direct protein/protein interaction and that this interaction generates α-migrating, nascent HDL particles containing many phospholipidated apoA-I molecules. In conclusion, the results presented here disprove the previous model. Rather, they suggest new concepts explaining the formation of nascent HDL particles and provide new avenues for the understanding of cholesterol homeostasis.

Key words : cardiovascular, cholesterol, efflux, HDL, ABCA1, apolipoproteinA-I.

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Abréviations utilisées dans cet ouvrage

22OH : 22(R)-hydroxycholesterol
2D-PAGGE : two-dimensional polyacrylamide non-denaturing gradient gel electrophoresis
9CRA : 9-*cis* retinoic acid
ABCA1 : ATP-binding cassette transporter A1
ACAT : acétyl:cholestérol acyltransférase
ACEH : acid cholesterol hydrolase
Apo : apolipoprotein
ATP: adenosine triphosphate
BSA : bovine serum albumine
BAC : bacterial artificial chromosome
BRET : bioluminescence resonance energy transfer
cAMP : cyclic adenosine monophosphate
CE : cholesteryl ester
CETP : cholesteryl ester transfer protein
CFTR : cystic fibrosis transmembrane conductance regulator
Chol : cholesterol
DMPC : dimyristoylphosphatidylcholine
DR : direct repeat
DSP : dithiobis(succinimidylpropionate)
DTNB : 5,5-dithiobis-2-nitrobenzoic acid
DTT : dithiothreitol
FBS : fetal bovine serum
FC : free cholesterol
FHD : familial HDL deficiency
FRET : fluorescence resonance energy transfer
GFP : green fluorescence protein
HDL : high density lipoprotein; lipoprotéine de haute densité
HDL-C : high density lipoprotein-cholesterol
HL : hepatic lipase
HMG-CoA : hydroxymethylglutaryl co-enzyme A
HSF : human skin fibroblasts
HSP : heat shock protein
H-TGL : hepatic lipase
ICC : intracellular compartments
IDL : intermediate density lipoprotein; lipoprotéine de densité intermédaire
LCAT : lecithin:cholesterol acyltransferase
LDL : low density lipoprotein; lipoprotéine de faible densité
LDL-C : low density lipoprotein-cholesterol
LDLR : LDL receptor
LpA-I : nascent apoA-I-containing particle
LPC : lysophosphatidylcholine
LPDS : lipoprotein deficient serum
LPL : lipoprotéine lipase

LXR : liver X receptor
MTP : microsomal triglyceride transfer protein
NBF/NBD : nucleotide binding fold; nucleotide binding domain
PC : phosphatidylcholine
PC-PLC : phosphatidylcholine-specific phospolipase C
PE : phosphatidylethanolamine
PFO : perfluoro-octanoic acid
PI : phosphatidylinositol
PL : phospholipids
PM : plasma membrane
PMSF : phenylmethylsulfonide fluoride
POPC : palmitoyloleylphosphatidylcholine
PPAR : peroxisome-proliferator activated receptor
PVDF : polyvinylidene fluoride
RCT : reverse cholesterol transport
r(LpA-I) : reconstituted HDL particles
RXR : retinoic X receptor
SDS-PAGGE : sodium dodecylsulfate-polyacrylamide gradient gel electrophoresis
SM : sphingomyelin
SM-ase : sphingomyelinase
SR-BI : scavenger receptor class B type I
SRE : sterol response element
SREBP : SRE-binding protein
TAP : transporter associated with antigen protein
TD : Tangier disease
TG : triglycéride
TLC : thin layer chromatography
TM/TMD : transmembrane domain
UC : unesterified cholesterol
VLDL : very low density lipoprotein; lipoprotéine de très faible densité

Chapitre I

État actuel des connaissances

Introduction

Devançant le cancer, le sida et les morts violentes, les maladies cardiovasculaires sont la première cause de décès dans le monde [1]. Insuffisance coronarienne, infarctus du myocarde, thrombose, embolie cérébrale, artériosclérose et hypertension artérielle sont autant d'exemples qui affectent 13 à 14 millions d'adultes américains [2]. À lui seul, l'infarctus du myocarde en tue un demi-million chaque année.

Les facteurs de risque de développement de maladies cardiovasculaires sont bien connus. Tabagisme, sédentarité et mauvaise alimentation sont les facteurs comportementaux modifiables. Diabète, obésité et taux élevé de cholestérol sont des paramètres métaboliques mesurables prédisposant au développement de la maladie. Bien que l'ingestion de cholestérol comme tel ne semble pas directement reliée à une plus grande incidence de maladie cardiaque chez l'humain [3], l'étude de Framingham a démontré l'importance centrale des transporteurs de cholestérol, i.e. les lipoprotéines, sur le risque de développer la maladie cardiovasculaire [4]. Cette étude a démontré une forte corrélation entre le risque de développer la maladie et les lipoprotéines de faible densité (LDL), tandis qu'une relation inverse a été observée avec les lipoprotéines de haute densité (HDL) [5].

Les lipoprotéines sont le moyen par lequel l'organisme transporte dans un milieu aqueux (plasma) des lipides pourtant insolubles dans l'eau vers leurs sites d'utilisation. Abondamment décrites dans le passé, les lipoprotéines se divisent en cinq sous-groupes majeurs : les chylomicrons, les lipoprotéines de très faible densité (VLDL), les lipoprotéines de densité intermédiaire (IDL), les lipoprotéines de faible densité (LDL), et les lipoprotéines de haute densité (HDL). ***C'est à la genèse de ces dernières (HDL) que cette thèse est consacrée.***

Le contexte métabolique du rôle des HDL sera d'abord brièvement présenté, suivi d'une description plus exhaustive de leur biogenèse. Ceci inclut la formation des particules HDL naissantes et la régulation de la molécule clé dans ce processus : l'*ATP-binding cassette transporter A1* (ABCA1). Les travaux pour élucider son fonctionnement (sa régulation, son interaction avec son ligand et sa structure fonctionnelle) seront présentés. Finalement, le travail va se clore sur une évaluation de la contribution à l'avancement des connaissances dans le cadre d'une discussion autocritique suggérant aussi de nouvelles avenues.

I.1 Métabolisme des lipoprotéines

1.1 Préambule

La composition et le métabolisme des lipoprotéines ont déjà été revus abondamment et font d'ailleurs partie de tout bon livre de biochimie [6]. Dans le but de bien mettre en contexte l'importance du rôle métabolique des HDL, une description sommaire du métabolisme des lipoprotéines sera d'abord faite. Celle-ci sera ponctuée de quelques découvertes récentes. Ensuite, le sujet de la thèse étant le mécanisme de genèse des HDL par l'ABCA1, ces deux composantes seront revues plus en détail.

1.2 Composition des lipoprotéines

Les lipoprotéines, comme leur nom l'indique, sont composées de lipides et de protéines reliés par des interactions non-covalentes. Les lipides qui les composent sont soit neutres (cholestéryl ester et triglycérides), soit chargés (phospholipides et cholestérol). Les lipides neutres se retrouvent à l'intérieur de la particule afin de minimiser les interactions avec l'eau. À l'inverse, les lipides chargés sont des molécules amphiphiles et forment l'enveloppe permettant la solubilité de la lipoprotéine dans l'eau. Ils présentent à la surface leur groupement polaire, tandis que leur chaîne carbonée est orientée vers le centre de la lipoprotéine où s'effectuent les interactions hydrophobes de type «Van Der Waals » avec les lipides neutres. C'est donc la présence d'un moment dipolaire - voire même l'existence d'une charge nette (phosphatidylinositol) - qui orientera la tête du lipide vers la surface de la lipoprotéine (lipides polaires). À l'inverse, l'absence d'un moment dipolaire (lipides neutres) permettra l'empaquetage à l'intérieur de la lipoprotéine, à l'abri de l'eau. Un shéma de la structure générale d'une lipoprotéine est montré à la figure I.1.

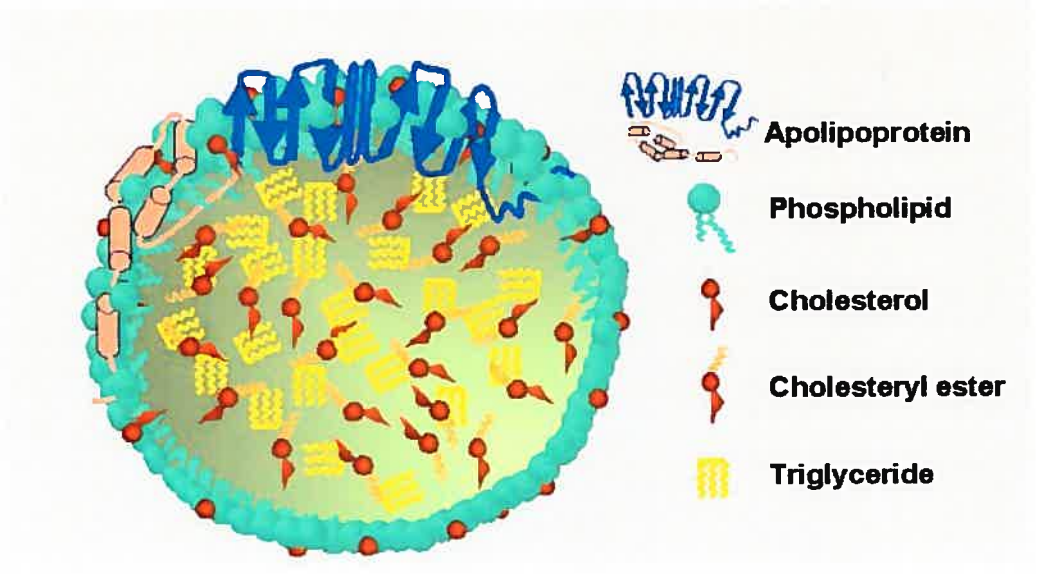

Figure I.1 : Schéma de l'organisation générale d'une lipoprotéine. Les lipides neutres (triglycérides et cholestéryl ester) se retrouvent au centre de la particule. Les lipides possédant un moment dipolaire (cholestérol et phospholipides) ou une charge nette négative font face au milieu aqueux. Les apolipoprotéines sont des molécules amphiphiles s'enchassant dans la particule et servant de cofacteurs pour les enzymes plasmatiques ou de ligand pour l'interaction avec des récepteurs spécifiques.

À part quelques autres protéines transportées par l'une ou l'autre des lipoprotéines, la constitution protéique est majoritairement assurée par les apolipoprotéines (apoprotéines). Ce sont des molécules amphiphiles. En effet, leur composition en hélices alpha ou en feuillets beta permet l'orientation des résidus chargés vers l'extérieur aqueux, tandis que les résidus hydrophobes sont orientés vers les lipides neutres, à l'intérieur. De cette façon, les interactions hydrophiles et hydrophobes sont maximisées. La nature amphiphile des apolipoprotéines les rend absolument essentielles au transport plasmatique des corps gras, insolubles dans l'eau. Finalement, chaque apolipoprotéine sert soit de co-facteur, soit de ligand pour un récepteur spécifique, permettant ainsi que chaque lipoprotéine assure le rôle qui lui est attribué.

Le contenu lipidique et apolipoprotéique de la lipoprotéine détermine sa classe et sa fonction. Chaque classe de lipoprotéine, son lieu d'origine et sa composition est détaillée à la table I.I

Table I.I Origine et composition des lipoprotéines

Lipoprotéine	Origine	Densité (g/mL)	Taille (nm)	Lipides majeurs	Apoprotéines majeures
Chylomicrons	Intestin	<0.95	100-1000	TG	B_{48}, AI, AII, AIV, E, CII, CIII
VLDL	Foie	<1.006	40-50	TG, CE, Chol	B_{100}, E,CII, CIII
IDL	VLDL	1.006-1.019	25-30	CE, TG	B_{100}, E
LDL	IDL	1.019-1.063	20-25	CE, TG	B_{100}
HDL	Foie, intestin	1.063-1.210	6-10	CE, Chol	AI, AII, E, CII, CIII

(TG = triglycérides (triacylglycérols), CE = cholesteryl ester, Chol = cholesterol)

On distingue trois voies principales de gestion métabolique des niveaux de cholestérol : la voie exogène, la voie endogène et le transport à rebours du cholestérol. La première est celle par laquelle le cholestérol entre dans la circulation par l'alimentation. Le cholestérol ingéré est micellisé par les acides biliaires et capté par l'intestin qui le transporte dans la circulation sous forme de chylomicrons vers le foie. La deuxième voie (endogène) est le résultat du contrôle de la formation des VLDL/IDL/LDL par le foie qui gère aussi le transport à rebours du cholestérol (« *reverse cholesterol transport* ») par les HDL. Dans le plasma, toutes ces particules peuvent interagir et échanger une partie de leur contenu à l'aide d'enzymes plasmatiques. Un schéma donnant un aperçu global du métabolisme des lipoprotéines est montré à la figure I.2.

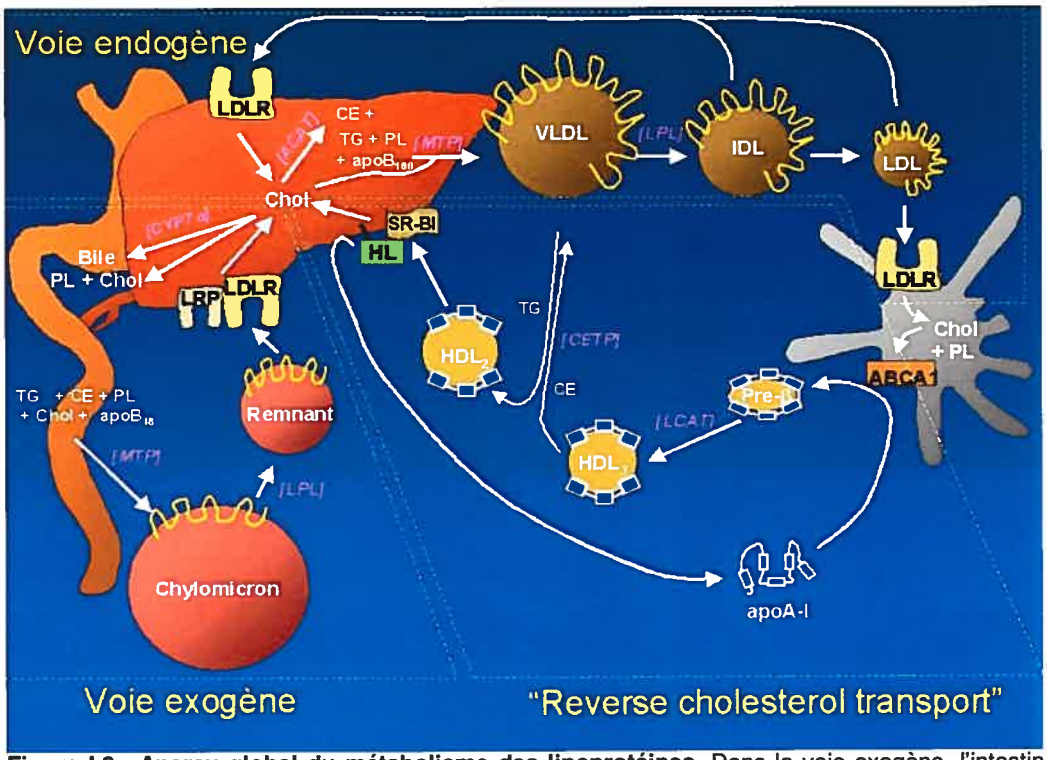

Figure I.2 : Aperçu global du métabolisme des lipoprotéines. Dans la voie exogène, l'intestin transfère les lipides de la diète micellisés par les acides biliaires dans la circulation sous forme de chylomicrons. Ces particules contienent l'apoB_{48} et sont enrichies en triglycérides (TG) et en cholestérol estérifié (CE). Leur enveloppe contient aussi des phospholipides (PL) et du cholestérol libre (chol). Elles sont le substrat de la lipoprotéine lipase (LPL) qui en fait des résidus (*Remnant*) captés par le foie via les récepteurs LDL et LRP. Dans la voie endogène, le foie produit lui aussi des particules riches en TG et en CE mais contenant l'apoB_{100} : les « very low density lipoproteins » (VLDL). La LPL et la protéine de transfert du cholesteryl ester (CETP) modifient ces lipoprotéines pour en faire des « intermediate density lipoproteins » (IDL). Celles-ci sont subséquemment transformées en « low density lipoproteins » (LDL), le cargo fournissant le cholestérol aux tissus périphériques (extrahépatiques). Ces derniers se débarrassent de l'excès de cholestérol par l'efflux via le transporteur « ATP-binding cassette A1 » (ABCA1) qui transfère des phospholipides et du cholestérol sur l'apoA-I pour former une particule pré-beta (pré-β) discoïdale. Ceci initie le processus de transport du cholestérol à rebours (« *Reverse cholesterol transport* »). Suivant la réaction de la lécithine:cholestérol acyltransférase (LCAT), la particule de « high density lipoprotein » (HDL) naissante (pré-β) devient plus sphérique et enrichie en CE (HDL_3). Le CE est échangé pour des TG par la protéine de transfert du cholestéryl ester (CETP) pour former les HDL_2 matures qui sont captées au foie par le récepteur vidangeur de type B1 (SR-BI). Ceci permet de transférer sélectivement le CE vers le foie, tandis que la lipase hépatique (HL) hydrolyse les TG.

1.3 Intestin et formation des chylomicrons

Suivant la prise d'un repas, les lipides de la diète sont solubilisés par la bile sécrétée par le foie dans le parcours gastro-intestinal. L'intestin joue un rôle critique dans la sélection des lipides à transférer vers la circulation. Récemment, cette fonction de tri assurée par l'intestin a été éminemment illustrée par l'identification des transporteurs ABCG5 et ABCG8 mutés chez les patients atteints de sitostérolémie. Ces deux hémitransporteurs hétérodimérisent afin de resécréter de l'épithélium intestinal les stérols végétaux (sitostérol, stigmastérol et campestérol) dans la lumière de l'intestin. Ainsi, l'endothélium intestinal de ces patients laisse anormalement passer les stérols qui sont donc transférés vers la circulation sanguine. En conséquence, ces patients souffrent d'hyperstérolémie importante conduisant au développement de xanthomes (dépôts de cholestérol dans la peau et les tendons) et à des maladies cardiovasculaires précoces. Contrairement aux autres types d'hypercholestérolémie, ces patients répondent très bien à une diète pauvre en cholestérol et à l'administration de résines retenant les composantes biliaires dans le tube digestif [7]. Aussi, l'ezetemibe, un médicament qui bloque l'absorption des stérols dans l'intestin, est d'un grand secours à ces patients.

Dans la physiologie normale, les lipides sélectionnés pour transfert du tube digestif vers la circulation sont captés puis assemblés autour de l'apoB_{48} dans l'épithélium de l'intestin par la «microsomal triglyceride transfert protein » (MTP). Cette dernière est une protéine du réticulum endoplasmique qui transfère les lipides absorbés vers l'apoB_{48} nouvellement synthétisée. Une fois l'assemblage terminé, des particules riches en cholestérol ester et en triglycérides appelées « chylomicrons » sont sécrétées dans la circulation lymphatique. En circulation, les chylomicrons deviennent le substrat de la lipoprotéine lipase (LPL). La LPL hydrolyse les triglycérides (triacylglycérols) contenus dans les chylomicrons en acides gras libres. La présence d'apoCII à la surface des chylomicrons l'active, tandis que l'apoCIII inhibe son activité. Les acides gras résultants de l'action de la LPL s'accumulent dans les adipocytes ou sont directement utilisés comme source d'énergie par les muscles squelettiques et le cœur [8]. La LPL transforme donc les chylomicrons en corps résiduels pauvres en triglycérides, mais riches en cholestérol esters. Le foie capte ensuite les résidus de chylomicrons via le récepteur LDL qui lie l'apoB et via d'autres récepteurs reconnaissant l'apoE. Le cholestéryl ester ainsi capté par le foie entrera subséquemment

dans la composition des VLDL ou sera hydrolysé pour servir de substrat à la synthèse des acides biliaires [9].

1.4 Lipoprotéines de très faible densité (VLDL)

Le foie joue un rôle central dans l'homéostasie du cholestérol. Il contrôle la voie endogène en sécrétant les « very low density lipoprotein » (VLDL). Dans le plasma, les VLDL acquièrent du cholestérol estérifié des HDL via la protéine de transfert du cholestéryl ester (CETP) avant d'être transformées par la LPL en IDL, qui deviendront finalement des LDL capables de fournir le cholestérol aux tissus périphériques. Assemblées un peu sur le même modèle que les chylomicrons, les VLDL contiennent toutefois une apoB complète, i.e l'apoB_{100}. Des études extensives sur la génération de ces particules ont permis de bien comprendre leur assemblage [10],[11]. En résumé, il est pré-requis à la production des VLDL que soient simultanément disponibles l'apoB_{100}, le cholestérol esterifié, les phospholipides et les triglycérides et une protéine facilitant l'empaquetage : la MTP.

Apolipoprotéine B_{100}

L'apoB_{100} est synthétisée de façon constitutive à un taux excédant celui de l'excrétion même des VLDL. La protéine d'apoB_{100} en surplus, non utilisée pour l'assemblage des VLDL, est rapidement dégradée. Dans le réticulum endoplasmique, la protéine s'associe de façon co-traductionnelle avec des lipides amphipatiques (cholestérol et phopholipides) lors de son passage à travers la membrane. À défaut de trouver des lipides avec lesquels s'associer de façon co-traductionnelle, l'apoB_{100} interagit avec la chaperone HSP70, devient ubiquitinée et est subséquemment ciblée au protéasome [12]. C'est la MTP qui facilite l'incorporation des triglycérides, des lipides neutres, dans la poche hydrophobe formée lors de la synthèse de l'apoB. La particule de VLDL ainsi formée est transportée vers l'appareil de Golgi et suit la voie de sécrétion normale vers la circulation sanguine (figure I.3).

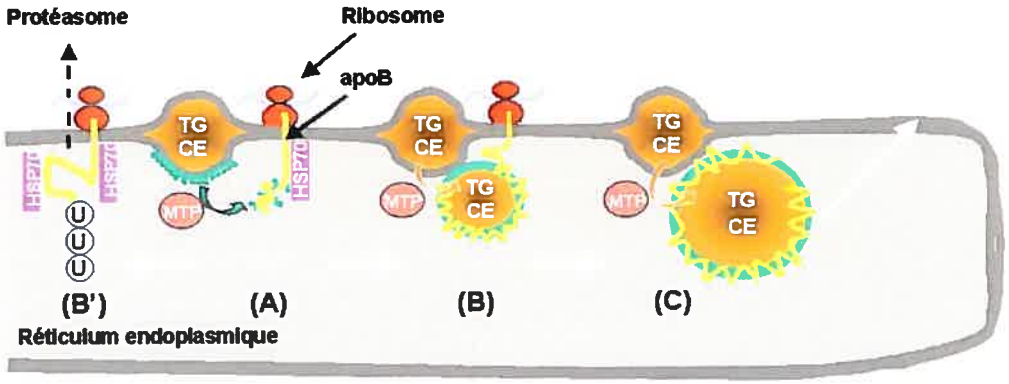

Figure I.3 : Assemblage des VLDL dans le réticulum endoplasmique de l'hépatocyte. (A) L'apolipoprotéine B_{100} ($apoB_{100}$) est synthétisée constitutivement et en excès dans le réticulum endoplasmique. Les lipides polaires comme le cholestérol (Chol) et les phospholipides (PL) ainsi que les lipides neutres **(B)** comme le cholestérol estérifié (CE) et les triglycérides (TG) sont transférés de façon co-traductionnelle par la protéine microsomale de transfert des triglycérides (MTP) sur l'apoB.**(C)** Ceci forme une particule VLDL mature qui est ensuite sécrétée dans la circulation. **(B')** La lipidation de l'$apoB_{100}$ par la MTP permet de diminuer les interactions avec la protéine du choc thermique (HSP70), évitant ainsi la dégradation protéosomique de l'$apoB_{100}$.

Biosynthèse du cholestérol

L'hépatocyte ne détient pas l'exclusivité de la biosynthèse du cholestérol et du cholestéryl ester, mais ce processus y joue un rôle majeur, car c'est un des facteurs limitants pour la production des VLDL. Si environ 50 enzymes au total sont impliquées dans la synthèse du cholestérol à partir de l'acétyl-Coenzyme A, l'étape régulatrice est la conversion de l'hydroxyméthylglutaryl-coenzyme A (HMG-CoA) en mévalonate par l'HMG-CoA réductase (figure I.4). Les produits de la réaction (mévalonate et cholestérol) exercent une rétroinhibition sur la transcription de l'enzyme. Au niveau post-traductionnel, la phosphorylation inhibe son activité enzymatique, alors qu'une forte concentration de stérols stimule sa dégradation. Finalement, l'enzyme peut être inhibée de façon compétitive par l'atorvastatine (Lipitor) ou autres statines possédant des constantes d'inhibition (Ki) de l'ordre du nanomolaire. Ces inhibiteurs sont couramment utilisés dans le traitement de l'hypercholestérolémie [13] afin de limiter la production des VLDL, diminuant ainsi le nombre de particules LDL circulantes.

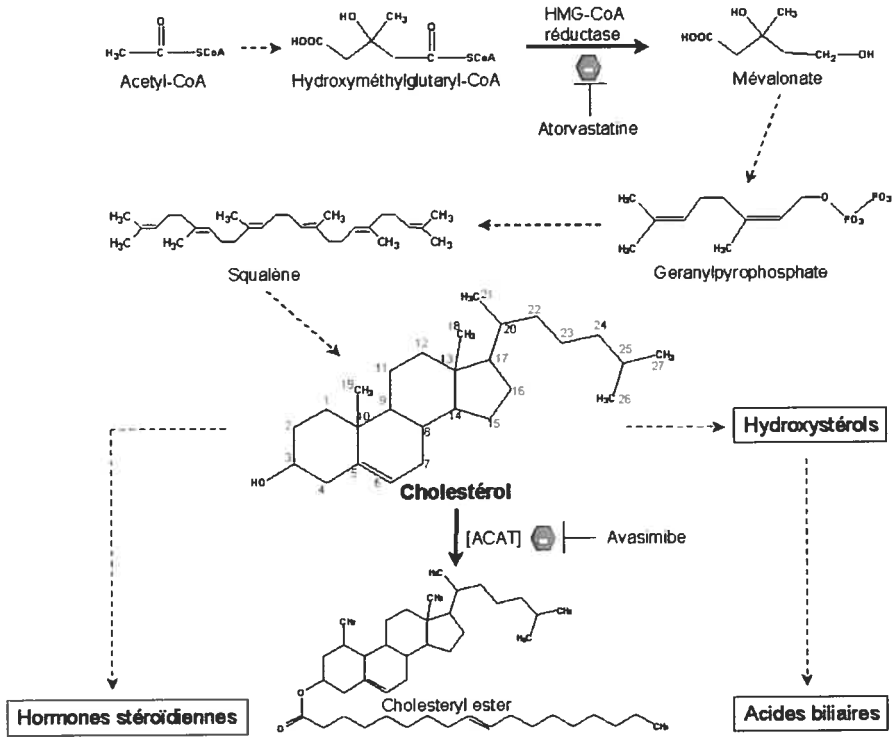

Figure I.4 : Étapes importantes dans la biosynthèse du cholestérol et du cholestérol estérifié. L'acétyl-CoA est modifiée en hydroxyméthylglutaryl-CoA servant de substrat à l'HMG-CoA réductase pour former le mévalonate. Cette enzyme régulatrice de toute la voie métabolique peut être inhibée par l'atorvastatine. Le mévalonate est transformé par une série de réactions enzymatiques pour former le géranylpyrophosphate. Cet intermédiaire entre dans la formation du squalène, le dernier intermédiaire avant la cyclisation de la molécule conduisant à la synthèse du cholestérol. Le cholestérol peut ensuite être estérifié par l'acyl:cholesterol acétyl transférase (ACAT), une enzyme inhibée par l'avasimibe. Les tissus stéroïdogéniques utilisent le cholestérol comme substrat pour la synthèse hormonale, tandis que le cholestérol peut aussi être transformé en hydroxystérols, les précurseurs des acides biliaires. Les flèches pleines représentent une réaction directe; les flèches hachurées signifient la présence de plusieurs étapes intermédiaires.

Formation du cholestérol estérifié

Toujours dans le but de limiter la production des VLDL, des inhibiteurs d'une autre enzyme, l'acyl-CoA:cholestérol acétyltransférase (ACAT), ont été développés. L'ACAT, une enzyme du réticulum endoplasmique catalysant l'estérification du cholestérol (figure I.4), est surexprimée en présence de cholestérol et/ou d'acides gras libres. Les inhibiteurs de l'ACAT comme l'avasimibe (CI-1011) fonctionnent au niveau cellulaire, mais les essais

cliniques ont démontré une efficacité modeste avec une certaine toxicité hépatique et surrénale. Par ailleurs, le développement des statines aptes à réduire la production de cholestérol sans effet secondaire majeur ont réduit considérablement l'intérêt à développer des inhibiteurs d'ACAT afin de contrôler la production des VLDL [14].

Nature des acides gras

Les acides gras libres sont les précurseurs de phospholipides et de triglycérides essentiels à la production des VLDL. La nature des acides gras influence l'empaquetage des VLDL. En effet, la culture de cellules hépatiques McArdle RH-7777 en présence d'acides gras polyinsaturés en position oméga-3 et en oméga-6 diminue la quantité de VLDL sécrétés [15]. Dans ce cas, la particule VLDL est assemblée normalement dans le réticulum endoplasmique, mais la forte présence d'insaturations des acides gras modifie l'empaquetage des VLDL et les redirige vers le lysosome pour dégradation. Ceci expliquerait en partie que les populations dont la diète est riche en poissons gras soient protégées des maladies cardiovasculaires. [16].

Protéine microsomale de transfert des triglycérides (MTP)

La protéine microsomale de transfert des triglycérides (MTP) fonctionne sous forme d'hétérodimère composé de deux sous-unités, l'une de 55kDa, l'autre de 97kDa. Cette protéine de la lumière du réticulum endoplasmique transfère les lipides sur la molécule d'apoB traduite, mais avec une meilleure efficacité pour les lipides neutres. Son rôle limitatif dans la production des VLDL a été mis en lumière par la découverte de mutations dans la sous-unité de 97kDa chez des patients atteints d'abetalipoprotéinémie (absence de lipoprotéines contenant l'apoB). Par ailleurs, l'inhibition de la MTP par « knock-out » ou par inhibition pharmacologique diminue les interactions de l'apoB avec les lipides amphipatiques, augmentant les interactions avec l'HSP70 qui la dirige vers la dégradation protéosomique [12],[17]. Un dysfonctionnement de la MTP résulte donc en une sous-production de VLDL.

1.5 Lipoprotéines de densité intermédiaire (IDL)

La lipoprotéine lipase (LPL) hydrolyse les TG contenus dans les VLDL sécrétées par le foie, ce qui les transforme en lipoprotéines de densité intermédiaire (IDL). Les IDL sont en fait des résidus de VLDL et contiennent donc moins de TG et sont enrichies en CE. Elles

contiennent aussi deux des apolipoprotéines (B_{100} and E) qui caractérisent les VLDL. Une partie des IDL nouvellement produites est reprise au foie via le récepteur LDL (LDLR) pour recycler les acides gras et regénérer des VLDL. L'autre partie des IDL sera le sujet de modifications métaboliques afin de générer des particules LDL riches en cholestérol ester et ne contenant que l'apoB_{100} [6].

1.6 Lipoprotéines de faible densité (LDL) et régulation périphérique

Les LDL sont considérées comme très athérogènes, car bien que les VLDL et les IDL soient athérogènes, leur demi-vie est neuf fois plus courte que celle des LDL. Aussi, plus les particules LDL sont petites et denses, plus leur pouvoir athérogène augmente, principalement parce que leur passage à travers les vaisseaux sanguins en est d'autant facilité [18],[19]. Une accumulation de LDL en circulation et dans la paroi artérielle due à une surproduction ou à une mutation dans le récepteur LDL (hypercholestérolémie) rend ces particules susceptibles d'être oxydées. Les macrophages de la paroi artérielle captent les LDL et se différencient en cellules spumeuses, cela modifie la texture de l'artère et enclenche des mécanismes de réparation par coagulation avec apparition subséquente d'un caillot.

Cependant, leur petite taille permet aussi aux LDL de jouer le rôle auquel elles sont « normalement » destinées : fournir le cholestérol aux tissus. Ce cholestérol capté est essentiel à maintenir l'intégrité membranaire ou est utilisé comme substrat dans la synthèse des hydroxystérols et des acides biliaires (foie). Goldstein et Brown ont démontré que la capture des LDL à la membrane plasmique s'effectue par le récepteur LDL (LDLR), une glycoprotéine trans-membranaire ayant une forte affinité pour l'apoB_{100} et l'apoE [20]. Le récepteur ainsi que la particule LDL sont alors emprisonnés dans les puits de clathrine qui s'invaginent et fusionnent avec le lysosome. À cet endroit, l'apoB_{100} est rapidement dégradée, les esters de cholestérols sont hydrolysés par l'hydrolase acide du cholestéryl ester (ACEH) en cholestérol qui peut être redirigé via les radeaux lipidiques (« rafts ») [21] vers la membrane plasmique, ou réestérifié et stocké dans le réticulum endoplasmique par l'acyl-CoA:cholestérol acyltransférase (ACAT). Le récepteur LDL, quant à lui, est recyclé à la membrane plasmique pour un nouveau cycle d'endocytose. Par ailleurs, la quantité de récepteurs LDL fonctionnels disponibles à la surface cellulaire constitue, avec la régulation de la synthèse *de novo* par l'HMG-CoA réductase, deux des

trois façons pour une cellule de contrôler sa quantité de cholestérol (figure I.5). Ce processus s'effectue via la protéine de liaison des éléments de réponse aux stérols (SREBP), un facteur de transcription activé par protéolyse en absence de cholestérol. Lorsque active, la SREBP est transloquée au noyau où elle stimule la transcription de l'HMG-CoA réductase et du récepteur LDL, en plus d'activer la transcription des gènes impliqués dans la synthèse des acides gras et des phospholipides [10].

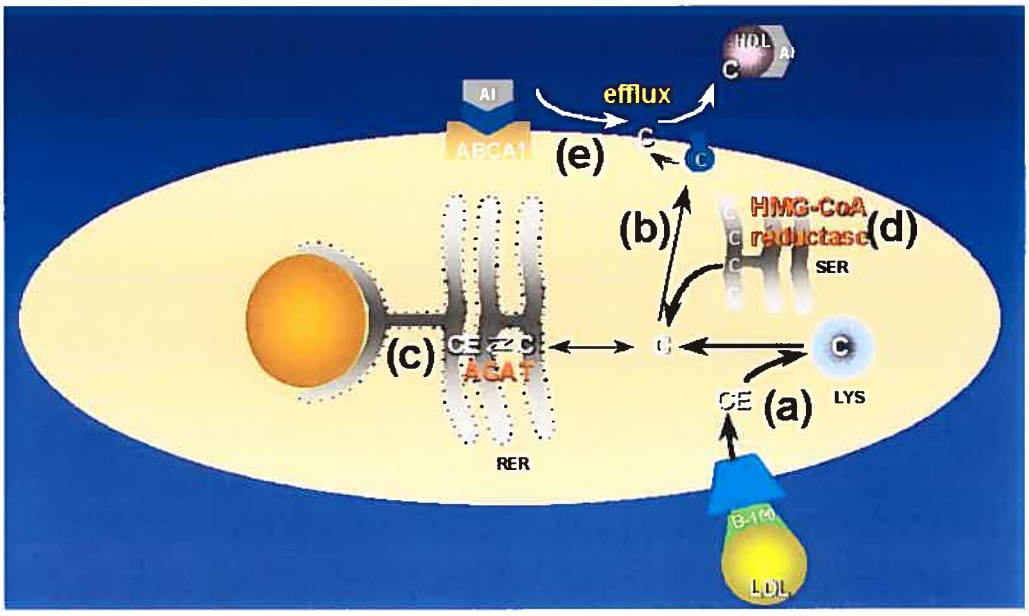

Figure I.5 : Homéostasie du cholestérol dans la cellule périphérique (extrahépatique). Les LDL circulantes sont captées par le récepteur LDL et invaginées dans les puits de clathrine **(a)**. Au lysosome, le cholestéryl ester (CE) est hydrolysé et transporté via les radeaux lipidiques (« rafts ») vers les *caveolae* **(b)**, ou transporté au réticulum endoplasmique pour être réestérifié par l'ACAT) **(c)**. Lorsque les niveaux cellulaires de cholestérol (C) sont trop bas, le récepteur LDL est surexprimé, simultanément à une biosynthèse *de novo* via l'activation de l'HMG-CoA réductase **(d)**. Le surplus de cholestérol doit être efflué par l'ABCA1 vers l'apolipoprotéine A-I (A-I) extracellulaire pour former des particules pré-β, i.e. des HDL naissantes **(e)**.

1.7 Lipoprotéines de haute densité (HDL)

Généralités

Les cellules périphériques (extrahépatiques) sont incapables de cataboliser leur cholestérol. Elles doivent donc utiliser une autre voie pour s'en débarrasser. Cette autre voie, c'est l'efflux de cholestérol et la formation de particules de haute densité (HDL), i.e. l'exportation de cholestérol vers l'extérieur de la cellule. S'il n'a jamais été cliniquement prouvé que des niveaux surélevés de HDL (hyperalphaprotéinémie) protègent contre la maladie cardiovasculaire, il n'y a aucun doute que des niveaux bas de HDL constituent un risque de développer la maladie coronarienne, et ce indépendamment des autres facteurs de risque [22]. Ceci est dû au fait que les HDL effectuent le transport du cholestérol à rebours (« reverse cholesterol transport »), c'est-à-dire le transport du cholestérol des tissus «périphériques » vers le foie [23]. Aussi, elles véhiculent le cholestérol ester vers les tissus stéroidogéniques comme les glandes surrénales et les organes reproducteurs où elles sont captées via le récepteur SR-BI. Les HDL ont un effet vasodilatateur en stimulant la production d'oxyde nitrique dans l'endothélium vasculaire. Enfin, les HDL transportent des molécules aux vertues antioxydantes comme la paraoxonase [24].

Métabolisme général

Contrairement aux autres lipoprotéines, les apoprotéines principales des HDL sont l'apoA-I et l'apoA-II. L'apoA-I synthétisée par le foie et l'intestin est sécrétée dans la circulation sous forme pauvre en lipides. *Le concept encore actuel* veut qu'au contact des tissus périphériques exprimant le transporteur « ATP-binding cassette A1 » (ABCA1), l'apoA-I acquière du cholestérol et des phospholipides pour former les particules appelées pré-β. Dans le plasma, les pré-β, de forme discoïdale, rencontrent la lécithine:cholestérol acyltransférase (LCAT). L'apoA-I servant de co-facteur, l'enzyme catalyse la trans-estérification d'un acide gras de la phosphatidylcholine (le phospholipide le plus abondant des HDL) au groupe 3-hydroxyl du cholestérol. Le cholestérol ainsi estérifié se déplace alors vers l'intérieur de la particule, générant un noyau central très hydrophobe et rendant la particule plus sphérique. Cette particule appelée HDL_3 rencontre alors la protéine de transfert du cholestéryl ester (CETP) qui transfère du cholestérol estérifié aux VLDL en échange de quelques triglycérides (figure I.2). La particule HDL_2 ainsi maturée est captée au foie par le récepteur vidangeur (scavenger) type B1 (SR-B1) qui permet l'ancrage des HDL_2 et le transfert sélectif du cholestérol estérifié vers l'hépatocyte. Pendant ce temps, la

lipase hépatique (HL) hydrolyse les triglycérides et les phospholipides. Le cholestérol transféré servira à la fabrication de la bile et des acides biliaires, qui seront ensuite recaptés dans l'instestin, bouclant le cycle entéro-hépatique. Les particules HDL sphériques soumises à l'action de la HL sont vidées de leur TG et redeviennent des particules pré-β discoïdales. La réduction de taille des HDL favorise le départ des apolipoprotéines qui se retrouvent pauvrement lipidées. Les apolipoprotéines dépourvues de lipides sont rapidement éliminées de la circulation par la cubiline, une protéine exoplasmique du rein qui, de concert avec la mégaline (une protéine de la famille des récepteurs LDL) [25], médie la racapture des apolipoprotéines libres et des HDL pour dégradation.

Origine tissulaire des HDL

Les macrophages accumulent des lipides dans la plaque athéromateuse. La forte expression d'ARNm d'ABCA1 dans ce tissus a soulevé la possibilité que les macrophages puissent contribuer d'une façon majeure à la quantité de HDL plasmatique. Pourtant, Haghpassand et coll. ont démontré le contraire. En effet, l'irradiation de souris sauvages et ABCA1 $^{-/-}$ suivie d'une retransplantation de moelle osseuse provenant de souris normales n'a pas augmenté les niveaux de HDL d'une façon significative [26].

Par contre, une surexpression adénovirale d'ABCA1 ciblée au foie a doublé les niveaux de HDL, suggérant un rôle central de cet organe dans la biogenèse des HDL [27]. Le foie est le tissus où l'ABCA1 et l'apoA-I sont les plus fortement exprimés. Afin de déterminer si l'ABCA1 de ce tissus pouvait contribuer à la genèse de HDL naissantes, Kiss et coll. ont mis en culture les hépatocytes primaires provenant de souris sauvages et ABCA1 -/-. Bien que l'absence d'ABCA1 n'empêche pas complètement la production hépatique d'apoA-I associée aux VLDL et aux HDL, elle diminue grandement la formation des HDL, confirmant un rôle central de l'ABCA1 hépatique dans la production des HDL plasmatiques [28]. Le foie a d'ailleurs été placé au centre de la figure I.6, afin d'illustrer le rôle central qu'il joue dans la biogenèse des HDL. Le mécanisme par lequel l'ABCA1 pourrait lipider l'apoA-I à l'intérieur de l'entérocyte n'est toutefois pas déterminé.

L'entérocyte produit aussi l'apoA-I, soit sous une forme délipidée, soit en association avec les chylomicrons. Toutefois, l'apport relatif de l'intestin à la formation des HDL plasmatiques n'a pas encore été évaluée.

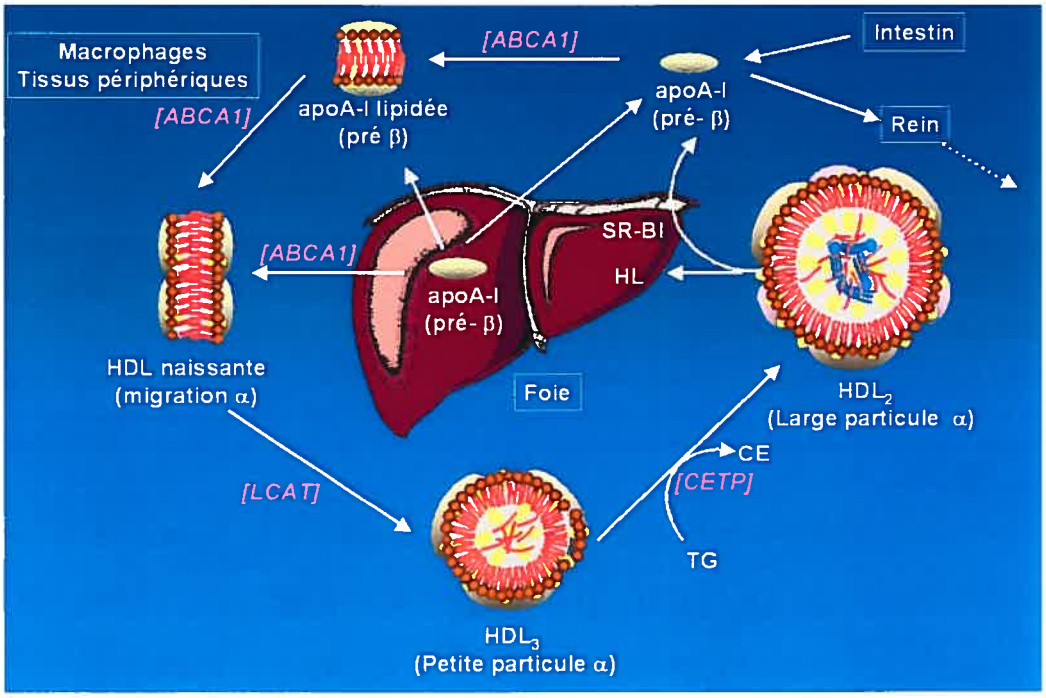

Figure I.6 : Origine tissulaire des HDL. Le foie joue un rôle central dans la biogenèse et le catabolisme des HDL. Il sécrète de l'apoA-I sous forme délipidée, sous forme lipidée et sous forme de HDL naissante. La forte expression d'ABCA1 dans ce tissus contribue d'une façon significative à la formation des HDL naissantes. L'intestin produit aussi l'apoA-I sous forme associée aux chylomicrons ou sous forme délipidée. Cet apoA-I devient lipidée au contact de l'ABCA1 des tissus périphériques et des macrophages, transformant ainsi des particules avec migration en position pré-β en particules HDL naissantes avec migration en position alpha. La LCAT estérifie le cholestérol des HDL naissantes pour former des HDL_3 sphériques. Ces dernières seront un substrat de la CETP qui échange des triglycérides (TG) pour du cholestéryl ester (CE) afin de former les HDL_2. Ces HDL_2 sont captés au foie par le récepteur SR-BI et la lipase hépatique (HL) et vident ainsi la particule de son contenu lipidique. Ceci libère de l'apoA-I libre et/ou pauvrement lipidée, complétant ainsi le cycle. L'apoA-I qui n'est pas lipidée est rapidement dégradée et excrétée de la circulation par le rein.

HDL et synthèse hormonale

Une portion du cholestérol estérifié des HDL sert à la synthèse des hormones stéroïdiennes. Au contraire des LDL qui sont complètement endocytées avec leur récepteur, les tissus stéroidogéniques captent les HDL à leur surface via le récepteur SR-BI. Seul le cholestérol estérifié est alors transféré vers la cellule, alors que le reste de la particule, incluant l'apoA-I demeure extracellulaire. Cela a été démontré d'une façon élégante en utilisant des HDL reconstituées avec du cholestérol éther radiomarqué, un analogue non hydrolysable de cholestérol ester ainsi que de l'^{125}I-apoA-I. Ce système a permis de montrer que le ^{3}H-cholestérol ether était transféré vers la cellule alors que l'^{125}I-apoA-I restait en surface [29]. Aussi, au fur et à mesure que les lipides sont tranférés vers la cellule, les apolipoprotéines sont libérées dans la circulation sous forme pauvre en lipides. En effet, le SR-BI a davantage d'affinité pour les HDL riches en lipides que pour les apolipoprotéines faiblement lipidées.

L'apolipoprotéine A-II et autres

Les HDL plasmatiques contiennent soit majoritairement l'apoAI, soit un mélange d'apoA-I et d'apoA-II dans un ratio molaire de 2:1 [30]. Comme l'apoA-I, l'apoA-II est sécrétée sous forme pauvre en lipides par le foie et constitue, après l'apoA-I, la protéine la plus abondante des HDL. Toutefois, le mécanisme par lequel l'apoA-II s'intègre aux HDL n'est pas clair. Si, comme l'apoA-I, l'apoA-II peut faire l'efflux des phospholipides et du cholestérol cellulaires, elle est incapable d'activer la LCAT et ne peut donc pas maturer en particules sphériques [31]. Ainsi, le transfert d'apoA-II des chylomicrons ou la fusion de particules contenant seulement l'apoA-I avec d'autres contenant seulement l'apoA-II sont deux voies possibles d'acquisition de l'apoA-II dans les HDL matures. Par ailleurs, d'autres apolipoprotéines mineures comme les apoC et l'apoE s'ajoutent aux HDL probablement par un mécanisme similaire [32].

Composantes métaboliques modifiant les HDL

a) Lécithine:cholestérol acyl transférase (LCAT)

La LCAT est une glycoprotéine de 49kDa sécrétée par le foie et les tissus adipeux et son association avec les HDL est influencée par les lipides et non par le contenu en apolipoprotéines de la particule. Son rôle est de transférer une chaîne acylée de la phosphatidylcholine (lécithine) sur le groupement OH du cholestérol. Elle joue un rôle

primordial dans la maturation des HDL d'une forme naissante et discoïdale (pré-β) vers une forme plus mature et sphérique (HDL_3). La LCAT requiert un co-facteur, l'apoA-I, et spécialement sa portion centrale, c'est-à-dire les acides aminés 143-187 (hélice 6) [33],[34],[35]. Les patients ayant une mutation dans le gène de la LCAT présentent la maladie appelée « Fish Eye disease » en raison de leur cornée anormalement opaque, une conséquence de l'accumulation de cholestérol et de phosphatidylcholine dans les membranes. Par ailleurs, d'autres patients ne présentent pas ce symptôme, mais dans tous les cas il y a absence de particules HDL matures dans le plasma (hypoalphalipoprotéinémie) (pour revue de la déficience en LCAT, voir références [36],[37]).

b) Protéine de transfert du cholestéryl ester (CETP)

Cette protéine de 476 acides aminés possède une poche hydrophobe permettant d'échanger du cholestérol estérifié pour des triglycérides entre les VLDL et les HDL. Dans le cas d'hypertriglycéridémie, cette fonction est stimulée, avec pour effet d'enrichir davantage les VLDL en CE et d'en réduire d'autant le contenu des HDL. Les particules HDL résultantes sont plus petites et ne sont pas retenues par le rein [38]. En conséquence le nombre de particules athérogènes (VLDL) augmente en même temps que la diminution des particules anti-athérogènes (HDL). Au contraire, les patients ayant une mutation de la CETP ont souvent des HDL élevés et des niveaux de LDL dans la normale. La sous-fraction HDL_2, la forme la plus enrichie en cholestérol estérifié, est très présente, tel qu'attendu. Cependant, certains chercheurs ont suggéré que malgré le grand nombre de particules HDL_2, ces dernières seraient dysfontionnelles et ne possèderaient pas de propriétés cardioprotectrices. Un débat similaire entoure le développement d'inhibiteurs pharmacologiques de la CETP [39]. Okamoto a démontré que l'inhibiteur JTT-705 réduisait considérablement l'athérosclérose chez le lapin [40]. Par contre, bien qu'un essai clinique ait montré qu'il permettait effectivement d'augmenter les HDL de 34% et diminuer les LDL de 7%, les auteurs de l'étude concluent que davantage de tests cliniques sont requis pour démontrer un bénéfice majeur sur la réduction de l'athérosclérose [41] chez l'humain. Plus récemment, le très prometteur Torcetrapid, un autre inhibiteur de la CETP, a augmenté de 61% les niveaux de HDL tout en réduisant les niveaux de LDL de 17% dans une étude réalisée chez l'humain [42]. Bien que les inhibiteurs de la CETP ouvrent une nouvelle voie de traitement possible afin d'améliorer la fonction vasculaire, plusieurs études sont encore requises afin d'établir les bénéfices de leur utilisation sur la santé humaine.

c) Lipase hépatique (HL)

Synthétisée par l'hépatocyte, cette glycoprotéine de 65kDa est sécrétée et demeure attachée à l'endothélium hépatocytaire. Elle hydrolyse des triglycérides, diglycérides et phospholipides et, en collaboration avec le récepteur SR-BI, elle facilite le transfert du cholestérol estérifié [43] vers l'hépatocyte. Les HDL ainsi dépourvues de lipides retournent en circulation sous forme de pré-β, tout en libérant leur contenu en apolipoprotéines libres. Comme pour la CETP, les mutations dans la HL résultent en une augmentation du nombre de particules HDL_2, sans toutefois clairement avoir d'effet athéroprotecteur.

d) Récepteur vidangeur de type BI (SR-BI)

Membre de la famille des récepteurs vidangeurs (scavenger), cette glycoprotéine de 80kDa (forme déglycosylée) lie plusieurs ligands comme les LDL, les HDL, les VLDL, les LDL modifiées et les apoprotéines [44]. Fortement exprimée au niveau du foie et des tissus stéroïdogéniques (surrénales, ovaires et testicules) [44], elle est aussi présente au niveau d'autres types cellulaires, notamment les macrophages de la plaque athéromateuse [45], sans toutefois y médier l'efflux de cholestérol [46]. En effet, contrairement à l'ABCA1, le SR-BI permet un transport bi-directionnel du cholestérol [47]. Les souris « knock-out » en SR-BI développent l'athérosclérose [48]. Récepteur liant aussi l'apoA-I, le SR-BI pourrait antagoniser l'action de l'ABCA1 dans les cellules exprimant les deux récepteurs en permettant l'influx de cholestérol provenant des particules dissociées d'ABCA1 [49].

I.2 Genèse et maturation des HDL

2.1 Caractérisation des particules

Même si la genèse et la maturation des HDL ont été revues abondamment [32],[50], il convient d'en faire une description plus détaillée. L'étude des particules HDL se fait souvent à partir de gels à deux dimensions, tel que décrits par Fielding [51]. Dans la première dimension, les composantes plasmatiques sont séparées selon **la charge** électrostatique dans un gel d'agarose. Les particules du plasma sont souvent identifiées selon leur position de migration dans ce gel. Ainsi, les particules les plus chargées migrent plus loin dans le gel, en position alpha. C'est le cas des HDL matures. En position intermédiaire migrent les particules moins densément chargées, à la position beta. C'est le cas des LDL. Les particules les moins chargées migrent en position gamma, c'est-à-

dire tout près de l'origine. C'est le cas des immunoglobulines et de la gamma-LpE, une particule dérivée des HDL ne contenant que l'apoE [52]. Entre ces positions (α, β, γ) se trouvent les régions pré-alpha et pré-beta. Les particules plasmatiques ainsi séparées font ensuite l'objet d'une deuxième séparation. La bande d'agarose contenant toutes les particules plasmatiques est coupée et placée au-dessus d'un gradient de 2-15% de gel de polyacrylamide en conditions non-dénaturantes. La migration se fait alors dans l'autre sens et la séparation s'effectue selon **la taille** de la particule. Le gel résultant peut alors être révélé à l'aide d'un anticorps anti-apoA-I, qui révèle ainsi toutes les particules contenant l'apoA-I trouvées dans le plasma. Un schéma d'une migration typique ainsi que d'un plasma normal est montré à la figure I.7. Ainsi, par gels à deux dimensions, Castro et Fielding ont identifié les particules appelées préβ$_1$, préβ$_2$, préβ$_3$, de même que les particules migrant en position alpha, les HDL$_2$, et HDL$_3$.

À l'aide de plasma incubé pendant une minute avec des cellules marquées au ^{3}H-cholestérol, Castro et Fielding ont proposé que ces particules auraient une relation précurseur/produit. En effet, les pré-β$_1$ (essentiellement de l'apoA-I peu ou pas lipidé) se sont enrichies en ^{3}H-cholestérol dans la première minute. Puis, lors d'une incubation en présence de cellules non-radiomarquées (chase), le marqueur tritié se retrouvait ensuite dans la fraction pré-β$_2$, et finalement dans les particules alpha. Le gel à deux dimensions, selon Castro et Fielding, permettrait donc d'avoir une photo instantanée de toutes les étapes intermédiaires de la maturation des HDL [53].

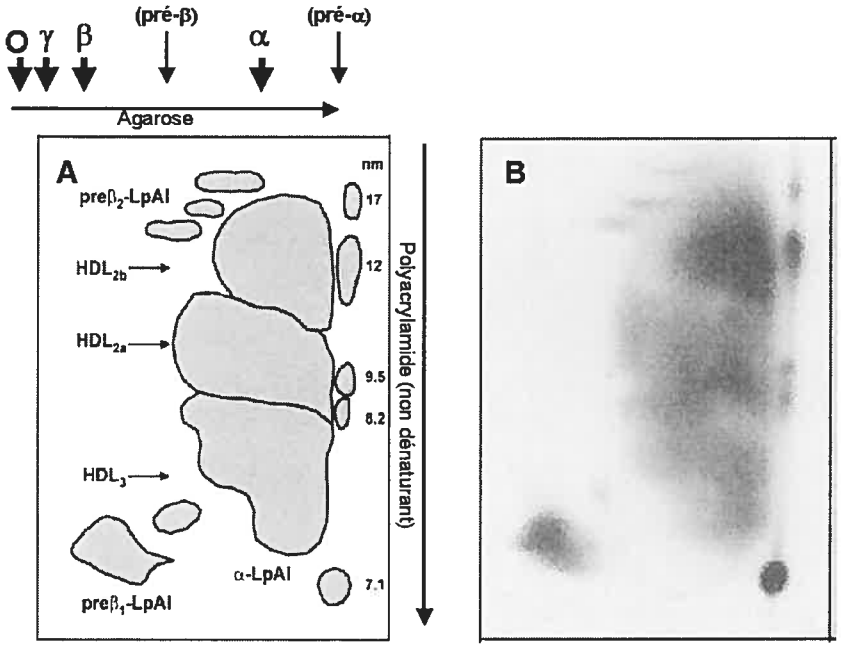

Figure I.7 : Migration des particules contenant l'apoA-I dans un gel à deux dimensions. A) Représentation schématique d'une migration typique sur gel à deux dimensions. Les composantes du plasma sont d'abord séparées dans une première dimension sur gel d'agarose, d'où l'on distingue la position alpha (α), beta (β) et gamma (γ), tout près de l'origine (**O**). La bande d'agarose contenant le plasma séparé est ensuite déposée au-dessus d'un gradient de gel d'acrylamide en conditions non-dénaturantes afin de séparer les composantes selon la taille. Le gel est ensuite transféré sur une membrane et on procède à la détection immunologique (Western) à l'aide d'un anticorps anti-apoA-I. **B)** Migration des particules contenant l'apoA-I du plasma d'un sujet contrôle. Adapté avec permission de référence [54]

Castro et Fielding ont défini que les pré-β_1 représentent une apoA-I peu lipidée, et probablement sous forme dimérique (60-75 kDa). Elles constitueraient donc un bon substrat pour une première capture de lipides. Toutefois, cette définition est sujet à la controverse. En effet, l'apoA-I complètement délipidée migre aussi à la position pré-β1 [55]. La limite de résolution du gel rend donc très difficile la distinction entre les particules discoïdales contenant 2-3 molécules d'apoA-I associées à des lipides *versus* l'apoA-I non-lipidée et mono-moléculaire.

Les pré-β_2 sont plus larges (~ 325 kDa) et constitutées essentiellement d'apoA-I dimérique avec un peu de lipides, notamment du cholestérol libre.

Les pré-β_3, invisibles dans la figure I.7, ne contribueraient qu'à une infime fraction de la quantité plasmatique totale d'apoA-I. Toutefois, des expériences de co-localisation ont révélé que 50% de la LCAT se retrouve dans cette particule, l'autre moitié étant partie intégrante des particules alpha [56]. Dans un système similaire à celui de Castro et Fielding (pulse-chase), Francone et collaborateurs ont démontré que les pré-β_3 étaient la première particule à générer du cholestérol estérifié. Un blocage subséquent de la LCAT par l'acide dithiobis(2-nitrobenzoïque) (DTNB) a permis de déterminer que le cholestéryl ester ainsi généré était transféré vers les particules alpha. Il a donc été conclu que les pré-β_3 étaient un état de transition où des préβ_2 entrent en contact avec la LCAT. Par ailleurs, Krimbou et collaborateurs ont clairement démontré par co-localisation que l'α_2-macroglobuline, une anti-protéase plasmatique, était intimement liée à la LCAT dans les pré-β_3. L'α_2-macroglobuline activée est un excellent ligand pour le récepteur LRP et l'association LCAT/α_2-macroglobuline médierait, via captation par le récepteur LRP, la dégradation de la LCAT [57].

Finalement, en position alpha migrent les particules HDL_3, qui sont plus sphériques que les pré-β et enrichies en cholestérol ester, tandis que les HDL_2, sphériques elles aussi, contiennent en plus des triglycérides (voir figure I.2), ce qui les rend moins denses. Les particules alpha sphériques transportent plus de 90% de l'apoA-I circulante [58]. Enfin, elles sont composées de deux, trois ou quatre molécules d'apoA-I ou d'un mélange d'apoA-I et d'apoA-II [59].

2.2 Apolipoprotéine A-I

Structure primaire et secondaire

L'apoA-I est synthétisée sous la forme d'une proprotéine de 249 acides aminés par le foie et l'intestin sous forme délipidée. Une fois en circulation, les 6 premiers résidus sont clivés, pour former une protéine pleinement active de 243 acides aminés pour une taille de 28.3 kDa. Elle joue un rôle crucial dans la détermination de la structure des HDL. [60]. On estime que dans sa forme délipidée, environ 55% de la protéine se trouve sous la

forme d'hélices alpha contre 75% lorsque associée à des lipides. Le segment à l'extrême N-terminal (1-43) serait composé d'une seule hélice alpha (résidus 8-32) suivi d'une chaîne peptidique flexible (résidus 33-44). Cette portion de la protéine adopterait une structure globulaire. L'analyse de la structure primaire de la partie C-terminale de l'apoA-I a prédit 10 hélices alpha séparées par des prolines. La structure secondaire serait donc composée de huit 22-mer et de deux 11-mer d'acides aminés (voir figure I.8) répétés en tandem. Chacune de ces séquences répétées ont la périodicité d'une hélice alpha amphipatique permettant l'insertion de la protéine dans une interface phospholipides/eau. Lorsque la protéine est mise en présence de lipides, la partie N-terminale de la protéine (résidus 1-43) reste sensible à une dégradation protéolytique, suggérant que cette partie ne participe pas à l'association de la molécule avec les lipides. Par contre, dans la partie C-terminale, les résidus 44-65 (hélice 1) de même que les résidus 220-241 (hélice 10), des régions riches en résidus aromatiques (Trp, Tyr et Phe) [61], sont celles possédant la plus forte affinité pour les lipides. Il a en effet été démontré à l'aide de vésicules de phospholipides en solution aqueuse (PBS) que les peptides synthétiques correspondant aux hélices 1 et 10 étaient les seuls à clarifier considérablement la turbidité de la solution en solubilisant les vésicules [62]. Par ailleurs, la partie centrale de l'apoA-I (hélice 6; a.a. 143-164), une région riche en arginines et dépourvue de résidus aromatiques [63], serait activatrice de la LCAT [64,65]. Récemment, en utilisant un peptide composé uniquement des hélices 1 et 9, ainsi qu'un peptide combinant les hélices 9 et 10, Natarajan et collaborateurs ont proposé que l'hélice 9 était essentielle dans la liaison de l'apoA-I avec l'ABCA1, tandis que les hélices 1 et 10 seules permettent l'efflux de lipides [66], sans permettre la liaison avec l'ABCA1.

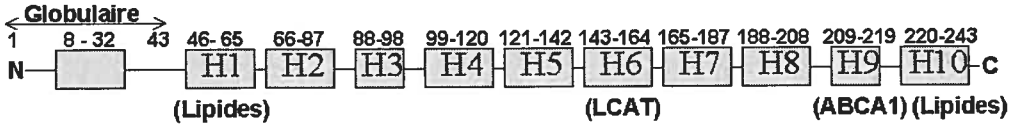

Figure I.8 : Structure secondaire de l'apoA-I. Selon les données prédites et les résultats empiriques, la partie N-terminale de l'apoA-I entrerait dans la formation d'un domaine globulaire. Le domaine C-terminal contient 10 hélices alpha qui formeraient une structure tertiaire en forme de torsade. Les hélices 1 et 10 ont la plus forte affinité pour les lipides, tandis que l'hélice 6 est requise pour l'activation de la LCAT. L'hélice 9, en combinaison avec les hélices 1 ou 10, permet la liaison à l'ABCA1 et l'efflux de lipides.

L'apoA-I libre s'autodimérise à forte concentration (> 0.1mg/ml) en solution aqueuse et une resolubilisation à la guanidine 4-6 M est requise afin de regénérer la forme monomérique [67]. Il a été suggéré que la forme monomérique en solution soit peu repliée et adopte une conformation semblable à une ellipse allongée d'environ 2.5 X 12.5 nm [68]. Cette conformation flexible permet aux domaines hydrophobes exposés de l'apoA-I d'interagir rapidement avec les lipides pour former une structure discoïdale. Il est possible par la technique du cholate de sodium de reconstituer *in vitro* ces particules HDL discoïdales à l'aide de POPC (palmitoyloleylphosphatidylcholine) ou de DMPC (dimyristoylphosphatidylcholine). Les particules ainsi formées sont composées de 160 molécules de phospholipides et de 2 molécules d'apoA-I, dont l'organisation quaternaire est l'objet de nombreux débats.

Structure tertiaire et quaternaire

Basés sur des observations au microscope électronique, Tall et coll. ont suggéré en 1977 que deux molécules d'apoA-I s'organisaient sous forme de piquets de clôture (« picket fence ») autour d'une bicouche de phospholipides. Ce premier modèle avait l'avantage que les résidus prolines interrompant les hélices alpha marquent bien les tournants dans la structure. De plus, la longueur de chaque hélice (~ 22 résidus) correspond à la longueur moyenne du domaine transmembranaire d'une protéine associée à une membrane. Les deux molécules d'apoA-I pourraient être orientées de façon tête-à-tête, ou tête-à-queue (figure I.9 A).

Toutefois, des données plus récentes de cristallographie et de diffraction des rayons X sur l'apoA-I Δ1-43 ont révélé une structure complètement différente. Basé sur ces données et sur une modélisation théorique maximisant les interactions salines interchaînes tout en orientant les résidus hydrophobes vers la bicouche de phospholipides, Segrest a proposé un modèle fort intéressant d'une structure en double ceinture (« double belt ») (figure I.9 B). L'apoA-I y serait arrangé sous forme de deux torsades antiparallèles. Cette structure thermodynamiquement favorable respecte les dimensions empiriques d'une particule discoïdale reconstituée. Un problème toutefois subsiste : il a été rapporté que des particules discoïdales comportant 3 molécules d'apoA-I existent, alors que le modèle de la double ceinture de Segrest peut difficilement accommoder une troisième molécule d'apoA-I.

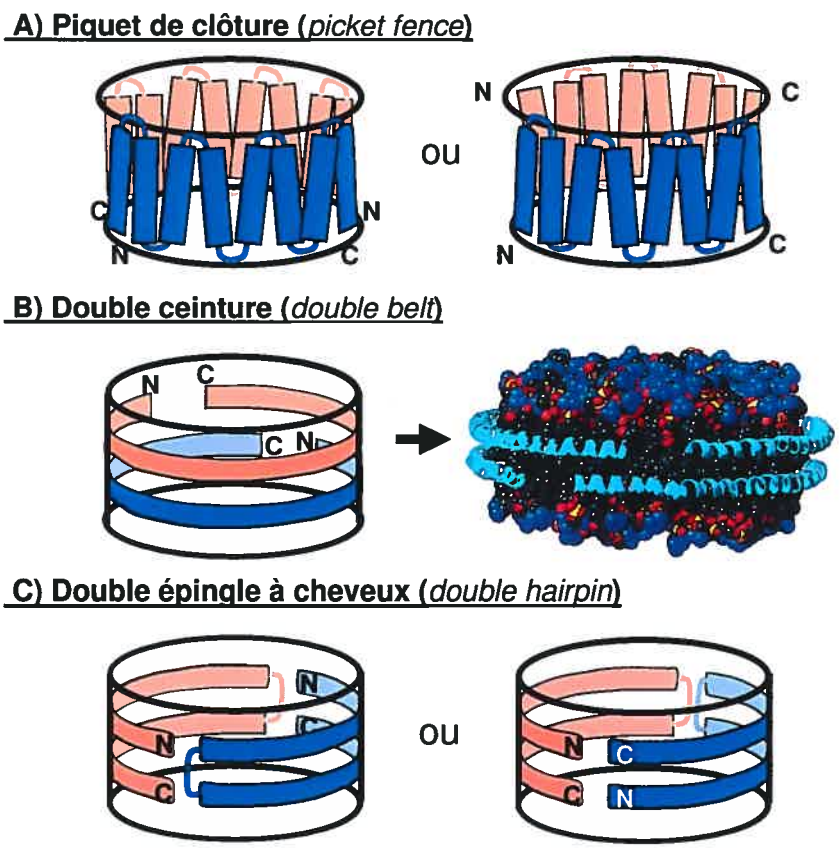

Figure I.9 Structures tertiaire et quaternaire de l'apoA-I. Trois modèles ont été proposés pour l'organisation de l'apoA-I dans les particules discoïdales. A) L'organisation en piquet de clôture a d'abord été suggéré par Tall et collaborateurs sur la base d'observations au microscope électronique. Ce modèle se base sur l'idée que les hélices alpha sont orientées d'une façon antiparallèle, comme pour un domaine transmembranaire. B) Le modèle de la double ceinture proposé par Segrest et collaborateurs est basé sur des données cristallographiques et de modélisation informatique et prévoyant la maximisation des interactions salines interchaînes combinée à une orientation forcée des résidus hydrophobes vers l'intérieur de la particule. Un modèle en présence de phospholipides est aussi présenté (à droite) C) Le modèle en épingle à cheveux est actuellement favorisé, car en plus des interactions interhélices conservées du modèle de la double ceinture, ce modèle permet d'accommoder une troisième molécule d'apoA-I, comme il est possible d'en générer. (Adapté de [69] et [70])

Enfin, à l'aide d'études de transfert d'énergie de fluorescence (« fluorescence resonance energy transfer », FRET), Tricerri a calculé une plus grande proximité des résidus alanine 124 et 232 que ce qui était prédit par le modèle en double ceinture. Cela suggérait une

interaction plutôt intramoléculaire. Un troisième modèle a ainsi été proposé, celui de l'épingle à cheveux [69]. Deux molécules d'apoA-I s'organiseraient dans une orientation tête-à-tête, ou dans une orientation tête-à-queue (figure I.9 C). Dans ce modèle, chaque apoA-I serait repliée sur elle-même, adoptant une structure en épingle à cheveux, avec un repliement à la glycine 129 au milieu de l'hélice 5. D'ailleurs, dans le modèle théorique de la double ceinture, l'hélice 5 était celle avec le moins d'interactions interhélices [71]. Ce modèle comporte plusieurs avantages. En effet, toutes les interactions *inter*moléculaires prédites par la cristallographie et la modélisation sont maintenues, à la différence qu'elles sont maintenant *intra*moléculaires. Il s'agit donc d'une structure (théoriquement) thermodynamiquement favorable. De plus, il permet d'expliquer aussi l'existence de particules composées de trois molécules d'apoA-I. Ces molécules pourraient être organisées avec les trois en épingle à cheveux; alternativement, une seule molécule pourrait être dans cette conformation, tandis que les deux autres pourraient former une double ceinture [71]. Enfin, Rogers a proposé que cette structure était celle qui expliquait le mieux la transition de l'état délipidé à l'état lipidé d'un seul monomère d'apoA-I [72]. En effet, dans cette conformation, l'hélice 1 interagit intramoléculairement avec l'hélice 10, créant ainsi un domaine composé des deux hélices ayant la plus forte affinité pour les lipides. Cette région lierait d'abord les lipides, permettant la réorganisation et l'enchassement, avec un autre monomère, dans l'environnement hydrophobe de la particule discoïdale. Toutefois, bien que fondée sur des observations empiriques, cette théorie reste à être démontrée.

Mutations de l'apoA-I

La majeure partie des mutations de l'apoA-I cause une hypoalphalipoprotéinémie due soit à une mauvaise synthèse, soit à une mauvaise interaction avec la LCAT. Deux mutants d'apoA-I donnent pourtant un phénotype inattendu : l'apoA-I_{Milan} (Arg173Cys) et l'apoA-I_{Paris} (Arg151Cys). La dimérisation par pont disulfure soudainement permise de ces molécules inhibe l'action de la LCAT. Pourtant, malgré que ces mutations causent un niveau très bas de HDL, elles semblent avoir un effet athéroprotecteur. Afin d'expliquer ce phénomène, Bielicki a utilisé un système de micelles de phospholipides et de déoxycholate exposées à la lipoxygénase, en absence ou en présence d'apoA-$I_{sauvage}$, apoA-I_{Milan} et apoA-I_{Paris}. Alors que les deux mutants ont la même capacité que l'apoA-$I_{sauvage}$ à faire l'efflux de cholestérol via l'ABCA1, ils avaient la capacité d'inhiber la peroxydation des lipides [73]. Au contraire des autres mutations qui ont un effet délétère,

ces deux mutants gagneraient en fonction par leur propriété athéroprotectrices d'antioxydant. Par ailleurs, Li et collaborateurs ont remarqué que l'apoA-I_{Milan}, lorsque complexée aux phospholipides, inhibe l'aggrégation plaquettaire [74].

Finalement, il a été suggéré d'utiliser ces formes mutantes d'apoA-I pour injecter à des patients atteints de problèmes cardiovasculaires. Récemment, 104 patients avec un athérome corronaire ont reçu des infusions intraveineuses d'apoA-I_{Milan} pendant 5 semaines, tandis que le volume de la plaque athéromateuse était mesuré par ultrason intravasculaire. La moyenne des résultats a révélé une diminution du volume de l'athérome de 14.3 mm^3 chez les sujets traités vs 2.9 mm^3 pour les sujets ayant reçu le placebo [75].

Déficience familiale en HDL et maladie de Tangier

Si plusieurs cas d'**hyper**alphalipoprotéinémie sont expliqués par des mutations dans la CETP, de la lipase hépatique (HL) ou du récepteur SR-BI et que plusieurs cas d'**hypo**alphalipoprotéinémie sont dus à des mutations dans la LCAT et dans l'apoA-I, un cas restait, jusqu'à tout récemment, inexpliqué : la maladie de Tangier.

Dans les années 60, de jeunes patients de l'île de Tangier aux Etats-Unis se sont présentés à leur médecin avec des amygdales de couleur orangée, des dépots de cholestéryl ester dans les tissus réticuloendothéliaux et des niveaux de LDL bas, mais surtout, des niveaux de HDL pratiquement indétectables (pour revue, voir [76]. Ce caractère semblait se transmettre d'une génération à l'autre d'une façon autosomale dominante sur cette île où la co-sanguinité existait parfois. Plus tard, des cas similaires de déficience familiale en HDL (FHD) sont identifiés. La transmission y est aussi autosomale dominante, mais le phénotype est plus modéré : seuls des niveaux bas de HDL sont observés. L'analyse de leur profil plasmatique par électrophorèse bidimentionnelle confirme leur hypoalphalipoprotéinémie (très peu de particules alpha) [54]. À l'aide d'isotopes stables, Batal et collaborateurs montrent que la biosynthèse de l'apoA-I est normale, mais que sa dégradation est accélérée chez ces patients [77]. Aussi, réalisées *ex vivo* sur des fibroblastes, les études d'efflux de cholestérol avec l'apoA-I révèlent que l'efflux est très bas chez les cellules de Tangier, alors qu'il est intermédiaire chez les FHD [78], [54].

Tirant avantage de l'étude de grandes familles, quatre groupes (incluant le nôtre) utilisent le criblage génomique, l'haplotypage et la saturation de l'haplotype pour cerner le gène responsable. C'est en 1999 qu'est finalement publié que l'ABC1 (ancien nom pour l'ABCA1) du chromosome 9q31 est muté dans la maladie de Tangier [79],[80],[81],[82] et chez les patients atteints de déficience familiale en HDL [83]. Dans le cas des Tangier, la mutation est homozygote ou hétérozygote composée, alors que chez les FHD un seul allèle porte une mutation. L'abolition de la traduction par l'utilisation d'une séquence antisens a montré que la protéine est impliquée dans l'efflux de cholestérol vers l'apoA-I [79]. Ainsi, un transporteur ABCA1 muté résulterait en une incapacité à former des particules HDL naissantes. L'apoA-I qui n'est pas lipidée est rapidement retirée de la circulation par le rein, ce qui expliquerait les niveaux très bas de HDL observés chez ces patients [84].

L'ABCA1 fait partie de la superfamille des ABC. Les protéines ABC sont des flippases/translocases qui permettent le transport ATP-dépendant de molécules diverses contre leur gradient de concentration (pour une excellente revue des transporteurs ABC, voir référence [85].

I.3 La famille des ABC

3.1 Évolution

Une liste des transporteurs ABC décrits chez l'humain ainsi que des maladies auquelles ils sont associés est présentée à la table 1.II. Des exemples d'ABC se retrouvent à plusieurs niveaux de l'évolution. Chez la bactérie *Salmonella Typhimurium*, l'histidine perméase est composée de 2 sous-unités HisP et des sous-unités membranaires HisQ et HisM. L'union de ces sous-unités forment un transporteur ABC fonctionnel [86]. Chez la levure, une série de transporteurs sont présents, dont le Ste6p de *Saccharomyces cerevisiae* impliqué dans le transport de la phéromone peptidique appelée « facteur a » responsable de la reproduction sexuée de cet organisme [87]. Chez le nématode *C. elegans*, la protéine *ced7* serait impliquée dans l'engouffrement des corps apoptotiques. Enfin, le génome de la mouche et de la souris présentent plusieurs gènes encodant des transporteurs ABC semblables ou équivalents à ceux identifiés chez l'humain.

3.2 Structure

Tous les transporteurs ABC sont composés de deux motifs structuraux arrangés différemment, selon la classe de transporteur. Le premier motif est un domaine transmembranaire (TM), généralement composé de 6 et 8 hélices alpha traversant la membrane d'une façon antiparallèle. Le deuxième motif est celui permettant la liaison et l'hydrolyse de l'ATP appelé « domaine de liaison aux nucléotides » (*nucleotide binding fold*; NBF). Ce motif situé du côté cytosolique comprend les séquences appelées « Walker A » (GX_4GKT/S, où X = n'importe quel acide aminé) et « Walker B » (Z_4D, où Z = résidu hydrophobe) ainsi qu'un segment appelé « signature » débutant généralement par la séquence LSGG. Ces trois séquences, en coordination avec un métal divalent comme le magnésium, constituent le domaine NBF et sont essentielles à la liaison des nucléotides [88]. Les motifs TM et NBF sont toujours arrangés en alternance. Les transporteurs ABC sont divisés en deux classes : les transporteurs complets arrangés TM-NBF-TM-NBF (ou l'inverse), et les hémitransporteurs qui ne comprennent qu'une seule série TM-NBF (ou l'inverse). Il est attendu que ces derniers dimérisent ou hétérodimérisent afin d'exercer leur fonction. Un exemple de la structure secondaire de trois transporteurs ABC est montré à la figure I.10. La structure tertiaire révélée par microscopie électronique suggère que l'interaction de deux domaines NBF est requise pour former un transporteur fonctionnel, tandis que les domaines TM s'assemblent pour former un pore traversant la membrane [89], [90] (voir figure I.11).

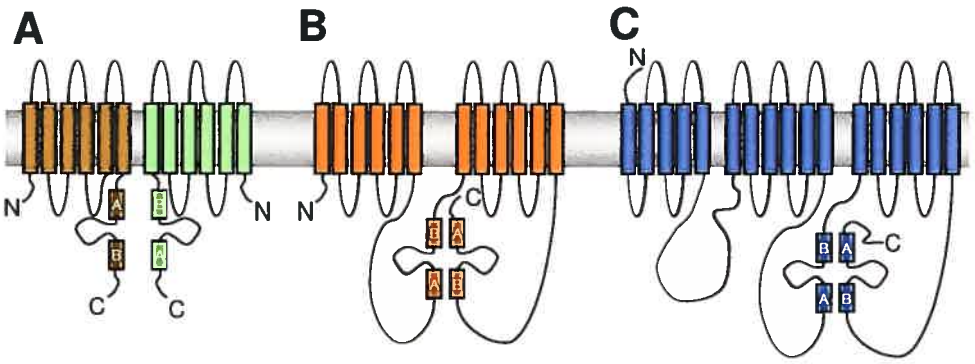

Figure I.10 : Arrangement de trois transporteurs ABC. Tous les ABC comprennent des domaines transmembranaires (TM) et des domaines de liaison aux nucléotides (NBF) arrangés en alternance. Le NBF comprend un domaine Walker A (A), un domaine Walker B (B), et un domaine appelé « signature » (S) (non-montré). **A)** Les deux hémitransporteurs ABCB2 et ABCB3 (TAP1 et TAP2) hétérodimérisent pour assurer leur fonction de transport des peptides antigéniques. **B)** La P-glycoprotéine est un transporteur complet impliqué dans la résistance aux drogues. **C)** Le transporteur ABCC1 (MRP1) est aussi impliqué dans la résistance aux drogues et possède des segments TM additionnels.

Pour ce qui est de la structure quaternaire, les hémitransporteurs forment soit des homo- soit des hétérodimères. C'est le cas des ABCG5/ABCG8 impliqués dans le transport des stérols et des transporteurs ABCB2/ABCB3 (TAP1/TAP2) impliqués dans le transport de peptides pour la reconnaissance immunitaire. Quelques cas d'homodimérisation de transporteurs complets ont aussi été rapportés. Ainsi, sur la base d'observations au microscope électronique en cryodécapage, Eskandari et collègues ont proposé que le transporteur ABCC7 (CFTR) impliqué dans la fibrose kystique formait un homodimère [91]. Par ailleurs, en utilisant des immunoprécipitations suivies de gels non-dénaturants, il a été possible de suggérer que l'hémitransporteur ABCG2 formait non seulement un homodimère, mais aussi un homotétramère [92]. Finalement, nous avons aussi démontré que la tétramérisation de l'ABCA1 constitue l'unité fonctionnelle minimale permettant la liaison du ligand, l'apoA-I (voir chapitre 5 ou référence [93]).

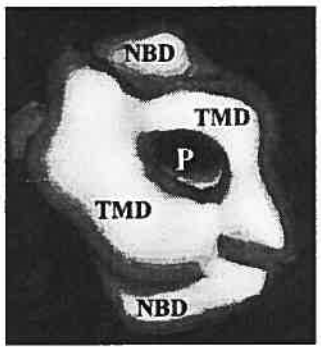

Figure I.11 : Projection en trois dimensions de la structure proposée du transporteur ABCB1 (Pgp). Les domaines transmembranaires (TMD) s'organisent pour former un pore (P) dans la membrane, tandis que les domaines de liaison aux nucléotides (NBD) sont cytosoliques. Tiré de la référence [90].

Table I.II : Transporteurs ABC connus

Symbole	Alias	Expression	Fonction	Maladie associée
ABCA1	ABC1	Ubiquitaire	Efflux PL, chol, PS, IL-1β	Tangier/FHD
ABCA2	ABC2	Cerveau	Résistance aux drogues	
ABCA3	ABC3	Poumon		
ABCA4	ABCR	Yeux, photorécepteurs	Efflux de rétinoides	Stagart
ABCA5		Muscle, cœur, testicules		
ABCA6		Foie		
ABCA7		Rate, thymus	Efflux de phospholipides?	
ABCA8		Ovaires		
ABCA9		Cœur		
ABCA10		Muscle, cœur		
ABCA12		Estomac		
ABCA13		Faible dans tous les tissus		
ABCB1	PGY1,MDR	Surrénales, rein, cerveau	Résistance aux drogues	
ABCB2*	TAP1	Toutes cellules	Transport peptidique	Infections virales
ABCB3*	TAP2	Toutes cellules	Transport peptidique	Infections virales
ABCB4	PGY3	Foie	Transp. Phosphatidylcholin	Cholestase hépatique
ABCB5		Ubiquitaire		Anémie sideroblast.
ABCB6*	MTABC3	Mitochondrie	Transport du fer	
ABCB7*	ABC7	Mitochondrie	Transport du fer/soufre	
ABCB8*	MABC1	Mitochondrie		
ABCB9*		Cœur, cerveau		
ABCB10*	MTABC2	Mitochondrie		
ABCB11	SPGP, MDR3	Foie	Acides biliaires	Cholestase hépatique
ABCC1	MRP1	Poumons, testicules	Résistance aux drogues	
ABCC2	MRP2	Foie	Efflux d'anions organiques	Dubin-Johnson
ABCC3	MRP3	Poumons, intestins, foie	Résistance aux drogues	
ABCC4	MRP4	Prostate	Transport des nucléosides	
ABCC5	MRP5	Ubiquitaire	Transport des nucléosides	
ABCC6	MRP6	Rein, foie		
ABCC7	CFTR	Tissus exocrines	Canal transport ion chlorure	Fibrose kystique
ABCC8	SUR	Pancréas	Récepteur de sulfonylurée	Hypoglycémie famil.
ABCC9	SUR2	Cœur, muscle		
ABCC10	MRP7	Faible dans tous les tissus		
ABCC11		Faible dans tous les tissus		
ABCC12		Faible dans tous les tissus		
ABCD1*	ALD	Peroxisomes	Ac. gras à longues chaînes	Adrénoleucodystrophie
ABCD2*	ALDL1, ALDR	Peroxisomes	Ac. gras à longues chaînes	
ABCD3*	PXMP1, PMP70	Peroxisomes	Ac. gras à longues chaînes	Zellweger
ABCD4*	PMP69, P70R	Peroxisomes	Ac. gras à longues chaînes	
ABCE1*	OABP, RNS4I	Ovaires, testicules, rate	Récepteur oligoadénylate	
ABCF1*	ABC50	Ubiquitaire		
ABCF2*		Ubiquitaire		
ABCF3*		Ubiquitaire		
ABCG1*	ABC8, white	Ubiquitaire	Efflux du cholestérol?	
ABCG2*	ABCP, MXR	Placenta, intestin	Toxines, résistance drogues	
ABCG4*	White 2	Foie	Efflux de cholestérol?	
ABCG5*	White 3	Foie, intestin	Transport des stérols	Sitostérolémie
ABCG8*		Foie, intestin	Transport des stérols	Sitostérolémie

* = hémitransporteur

I.4 Le transporteur « ATP-binding cassette A1 » (ABCA1)

4.1 Introduction

Le transporteur ABCA1 est encodé par 50 exons et la protéine compte 2261 acides aminés pour un poids moléculaire d'environ 240 kDa. Elle fut d'abord connue pour son rôle dans la phagocytose par les macrophages de cellules apoptotiques préalablement marquées au ^{51}Cr [94],[95]. Utilisant des macrophages murins surexprimant l'ABCA1, Hamon et coll. ont ensuite montré que le glyburide (glybenclamide), un médicament utilisé pour traiter l'hyperglycémie et inhiber les ABC, inhibait l'efflux d'interleukin-1β médié par l'ABCA1 [96],[97]. Puis, la propriété du transporteur de flipper la phosphatidylsérine en la redistribuant vers le feuillet externe de la membrane fut révélée à l'aide d'un essai de liaison d'annexine-V, une protéine liant la phosphatidylsérine [95]. Finalement, son identification comme gène responsable de la maladie de Tangier allait lui donner une vocation supplémentaire: l'efflux de cholestérol et de phosphatidylcholine vers l'apoA-I pour former des particules HDL naissantes.

Cette nouvelle affectation a été démontrée de plusieurs façons : 1) Les fibroblastes des patients FHD (hétérozygotes) et Tangier (homozygotes) effluent le cholestérol respectivement à environ 60% et 15% des valeurs normales [78], [54]; 2) Il y a corrélation entre les niveaux d'ARNm, la quantité de protéine ABCA1 exprimée et l'efflux de cholestérol [98]; 3) L'utilisation d'une séquence antisens dans des macrophages murins inhibe l'efflux de cholestérol médié par l'apoA-I [99]; 4) Le traitement de macrophages murins RAW à l'AMPc stimule la production d'ABCA1, en plus d'augmenter le « cross-linking » de l'apoA-I et l'efflux de cholestérol [100], alors que le glyburide inhibe l'efflux [101]; 5) la surexpression de l'ABCA1-GFP dans des cellules Hela augmente l'efflux de cholestérol et de phospholipides [95]; et 6) les souris « knock-out » pour l'ABCA1 présentent un phénotype de déficience en HDL [102] tandis que les souris transgéniques surexprimant la protéine ont un profil d'hyperalphalipoprotéinémie [103], [104].

L'identification de l'ABCA1 comme étant responsable de la liaison de l'apoA-I et de l'efflux de cholestérol a déclenché plusieurs études caractérisant sa régulation transcriptionnelle, sa régulation post-traductionnelle, et son interaction avec son ligand : l'apoA-I.

4.2 Régulation transcriptionnelle

La protéine est exprimée dans tous les tissus, avec une prédominance pour le foie, les testicules et les surrénales [105]. Sa transcription est sous le contrôle d'un promoteur principal et de trois promoteurs accessoires situés dans l'intron 1. (Voir figure I.12). Ainsi, les exons 1a/d, 1c et 1b ont été identifiés. C'est en effet à l'aide d'analyse d'ADNc générés dans des souris transgéniques utilisant des chromosomes artificiels bactériens (BAC) que Cavelier et coll. [106] ont identifié l'exon 1a ainsi que son promoteur. L'expression de ces vecteurs dans des souris transgéniques a révélé une expression tissulaire différente selon le promoteur utilisé. Ainsi, le promoteur pour l'exon 1 favorisait une expression dans les poumons et les surrénales, tandis que le promoteur attenant à l'exon1a favorisait une expression hépatique. Par ailleurs, à la recherche d'éléments DR4 (« direct repeat 4 ») fonctionnels dans l'intron 1, Singaraja et coll. y ont identifié trois régions promotrices suivies de leurs exons 1d, 1c et 1b (l'exon 1d de Singaraja correspond à l'exon 1a de Cavelier). L'utilisation de ces trois promoteurs dans des essais luciférase a révélé qu'ils étaient tous utilisables par les amplificateurs (« enhancer ») LXR/RXR pour activer la transcription [104]. Finalement, le patron de distribution de chaque ARNm a été étudié par RT-PCR (« real-time PCR ») plus en détail dans les différents tissus. Tandis que l'exon 1 est exprimé dans tous les tissus, les exons 1a/d, 1c et 1b sont plus présents dans le foie et les poumons [107]. Ceci suggère que l'utilisation de différents promoteurs permet une régulation tissulaire différentielle.

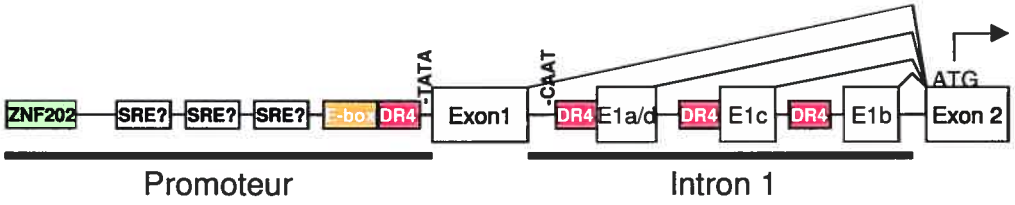

Figure I.12 : Éléments de régulation des promoteurs d'ABCA1. L'ABCA1 est sous le contrôle d'un promoteur principal et de trois promoteurs alternatifs situés dans l'intron 1. Les exons 1, 1a/d, 1c et 1b ne sont pas codants, le codon ATG de départ étant situé dans l'exon 2. Les différents exons 1 sont donc utilisés par différents types cellulaires sans atteinte à la structure primaire de la protéine. Les différents promoteurs contiennent les éléments de base (boîte TATA et boîte CAAT) en plus d'éléments putatifs de régulation par les stérols (SRE). Les éléments DR4 assurent la liaison des récepteurs LXR et RXR activant la transcription, tandis qu'un élément permet la liaison du répresseur ZNF202. La boîte E (E-box) permet la liaison des facteurs USF1 et USF2 avec le répresseur de transcription Fra-2. Cet élément serait utile lors de la mitose.

Les premières expériences de régulation génique réalisées par Langmann et collaborateurs ont montré que l'ABCA1 était surexprimée dans des macrophages après incubation avec des LDL acétylés, suggérant un contrôle par la quantité de stérols cellulaires [108]. Ceci est logique puisque le transporteur a pour rôle d'expulser le cholestérol vers l'extérieur de la cellule afin de maintenir l'intégrité membranaire. Ainsi, la séquence du promoteur principal de l'ABCA1 comporte des éléments de régulation spécifiques aux stérols comme les éléments DR4, SRE, un élément de liaison du répresseur ZNF202 et une boîte E (« E-box »).

Élément « direct repeat 4 » (DR4) et le « liver-X-receptor » (LXR)

Il est intéressant de noter que tous les promoteurs en amont des différents exons possèdent un élément DR4. Ceci est compatible avec le rôle primordial que jouent les stérols dans la régulation transcriptionnelle de l'ABCA1. L'élément DR4 est composé de la séquence AGGTCA répétée deux fois, mais séparée par 4 nucléotides. Il permet la liaison de l'hétérodimère « liver-X-receptor/retinoid-X-receptor » (LXR/RXR). Alors que le récepteur RXR a pour agoniste l'acide rétinoïque, le LXR est activé par les hydroxystérols comme le 22-R-hydroxycholestérol (22OH) et le 27-hydroxycholestérol (27OH). Lorsque ces deux récepteurs sont activés par leur ligand respectif, ils s'associent à l'élément DR4 et activent la transcription, un événement inhibé par le géranylgéranyl-pyrophosphate (GGPP) [109]. Outre l'ABCA1, l'élément DR4 est retrouvé dans le promoteur de gènes reliés au métabolisme du cholestérol comme la CETP, l'ABCG1, la SREBP, la CYP7α (synthèse des acides biliaires) [110], l'apoE, et le LXR lui-même [111]. L'élément DR4 et l'activateur LXR exercent un rôle régulateur central dans la transcription de l'ABCA1. La surexpression du LXR dans des fibroblastes et les macrophages et/ou le traitement avec des hydroxystérols augmente de 7 à 30 fois l'expression de l'ARNm d'ABCA1 [112]. À l'inverse, la mutation ou la délétion de l'élément DR4 entraîne une diminution dramatique de la réponse dans un essai luciférase [113],[114]. Enfin, les agonistes des facteurs PPAR (« peroxisome-proliferator activated receptors ») alpha et gamma activés par les acides gras et leurs métabolites activent la transcription d'ABCA1. Comme le promoteur d'ABCA1 ne possède pas d'élément de liaison des PPAR, Chawla et coll. ont plutôt proposé que les PPAR activent la transcription du LXR qui à son tour active la

transcription d'ABCA1 [115]. Un shéma de la régulation d'ABCA1 par les stérols est présenté à la figure I.13.

Éléments de réponse aux stérols (SRE)

Jusqu'à maintenant, aucun rôle exact n'est connu pour les éléments putatifs SRE. L'élément SRE (sequence consensus = CCACGCAAC) permet normalement la liaison de la SREBP qui active la transcription en absence de cholestérol. Il n'est pas exclu que la SREBP agisse comme répresseur transcriptionnel de l'ABCA1 en absence de stérols comme c'est le cas pour le promoteur de la MTP [116]. Cependant, jusqu'à maintenant aucune évidence ne suggère un rôle primordial des éléments SRE dans une régulation transcriptionnelle directe de l'ABCA1 par le cholestérol [117]. D'où vient alors la surexpression de l'ABCA1 en abondance de cholestérol? Nous avons proposé, comme d'autres groupes, que le cholestérol serait transformé en hydroxystérols, les activateurs du LXR [118]. En effet, les mutations dans la stérol-27-hydroxylase rendent les patients incapables de convertir leur cholestérol en 27-hydroxycholestérol, ce qui inhibe la transcription de l'ABCA1 et ce, même en abondance de cholestérol [119].

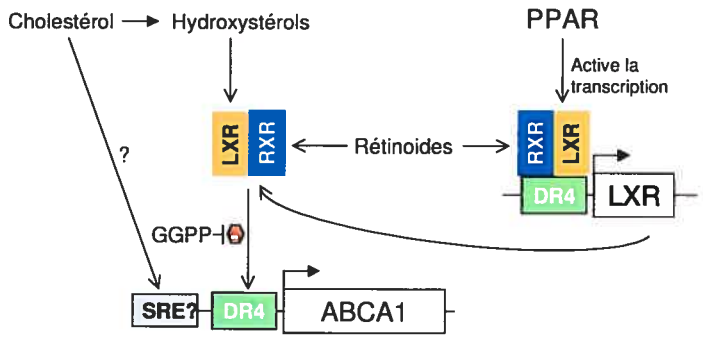

Figure I.13 : Régulation proposée d'ABCA1 par l'élément DR4. L'augmentation du niveau de cholestérol intracellulaire entraîne une conversion du cholestérol en hydroxystérols, des ligands du «liver-X-receptor» (LXR). Ce dernier hétérodimérise avec le «retinoid-X-receptor» (RXR) pour se lier à l'élément DR4 du promoteur de l'ABCA1. Cette interaction est inhibée par le géranylgéranyl-pyrophosphate (GGPP). La quantité de LXR intracellulaire, augmentée par l'activation des «peroxysome-proliferator activated receptors» (PPAR), détermine le niveau d'expression d'ABCA1.

ZNF202

Cette protéine nucléaire riche en doigts de zinc, majoritairement exprimée dans le cœur, le foie, les poumons et les testicules, est reconnue comme répresseur transcriptionnel se liant à la séquence consensus 5'-GGGGT-3'. Cette séquence est retrouvée dans le promoteur de nombreux gènes reliés au métabolisme du cholestérol comme certaines apolipoprotéines [120], la LPL, la LCAT et l'ABCG1 [121]. Son association avec le promoteur de l'ABCA1 a été démontrée, comme pour les autres promoteurs, par déplacement sur gel («gel-shift»). La délétion de la séquence consensus entraîne une surexpression dans un essai luciférase, tandis que la surexpression de ce facteur dans les macrophages murins RAW264 réduit l'efflux de phospholipides et de cholestérol. Enfin, une simple hybridation Northern a montré que la charge en cholestérol réduit l'expression de ce facteur, suggérant une relation inverse entre la régulation de l'ABCA1 et celle du ZNF202 [120]. Ceci ajoute aux autres éléments de régulation de l'ABCA1 par les stérols.

Boîte E

Lorsque cet élément est muté, la transcription augmente. Un essai luciférase, lorsque cette boîte est délétée, donne une surexpression par un facteur 3 dans les macrophages murins RAW. Ceci n'affectait en rien l'activation par du 22OH en combinaison avec l'acide 9-*cis*-retinoïque, suggérant que les deux mécanismes de régulation sont indépendants. Ceci suggère qu'un répresseur se lie à cette région et inhibe la transcription. En effet, la boîte E contient une séquence consensus de liaison pour USF1 et USF2, des facteurs de transcription de type hélice-boucle-hélice. Par empreinte génomique (footprinting), Yang et coll. ont montré que ces facteurs liaient la boîte E et activaient la transcription. Cependant un déplacement sur gel (gel shift) a révélé que le répresseur Fra-2 faisait aussi partie du complexe, ce qui inhiberait la transcription d'ABCA1 [122]. Fra-2 étant un proche parent de c-Fos, la boîte E serait donc impliquée dans le contrôle transcriptionnel lors de changements dans le cycle cellulaire (mitose).

AMPcyclique (AMPc)

Oram et coll. [100] ont démontré que l'AMPc induisait l'expression d'ABCA1 dans des macrophages murins RAW264. Même si aucun élément de régulation n'a formellement été identifié dans le promoteur d'ABCA1 pour expliquer cette régulation, Tamura et coll. ont montré que le LXRα est un régulateur transcriptionnel pouvant se lier à un élément

« CNRE » régulé par l'AMPc dans le promoteur de la rénine chez la souris [123]. Si ceci se produit chez l'ABCA1, ce pourrait représenter une avenue pour expliquer la régulation par l'AMPc. Par contre, nous avons montré (ainsi que Cavelier et coll. [106]) que l'effet inducteur de transcription de l'AMPc est observé seulement chez les macrophages de souris et non chez les macrophages humains.

Autres éléments

D'autres éléments de régulation sont présents dans le promoteur d'ABCA1. C'est le cas notamment de l'élément Ying-Yang1 (YY1) qui pourrait agir comme élément sensible au cholestérol. Aussi, plusieurs éléments SP-1 sont présents, des éléments « hepatic nuclear factor 3β » (HNF3β) et une séquence AP-1 [124]. Bien que par déduction on puisse leur supposer des fonctions, des études supplémentaires sont requises pour les confirmer.

4.3 Structure et régulation post-traductionnelle

Structure générale

L'analyse de la séquence primaire de l'ABCA1 prédit deux domaines transmembranaires (TM) comprenant chacun 6 hélices alpha orientées de façon antiparallèle, intercalés entre deux domaines liant les nucléotides (NBF). Un shéma de la structure secondaire de l'ABCA1 est montré à la figure I.14. La protéine est synthétisée avec un peptide-signal dont le clivage est incertain. Deux groupes, l'un utilisant une construction avec un épitope hémagglutinine (HA), l'autre avec la «green-fluorescence protein» (GFP) insérés en N-terminal ont montré des résultats contradictoires. Dans le premier cas, l'immunoprécipitation suivie d'un Western a suggéré que le peptide-signal est clivé [125]. Dans le deuxième cas, aucun produit de clivage du fragment N-terminal-GFP n'a été détecté [126]. S'il est vrai que le clivage générerait une topologie atypique pour un transporteur ABC de classe A, les auteurs ne peuvent cependant pas exclure que la GFP de la protéine chimérique puisse camoufler le site de clivage.

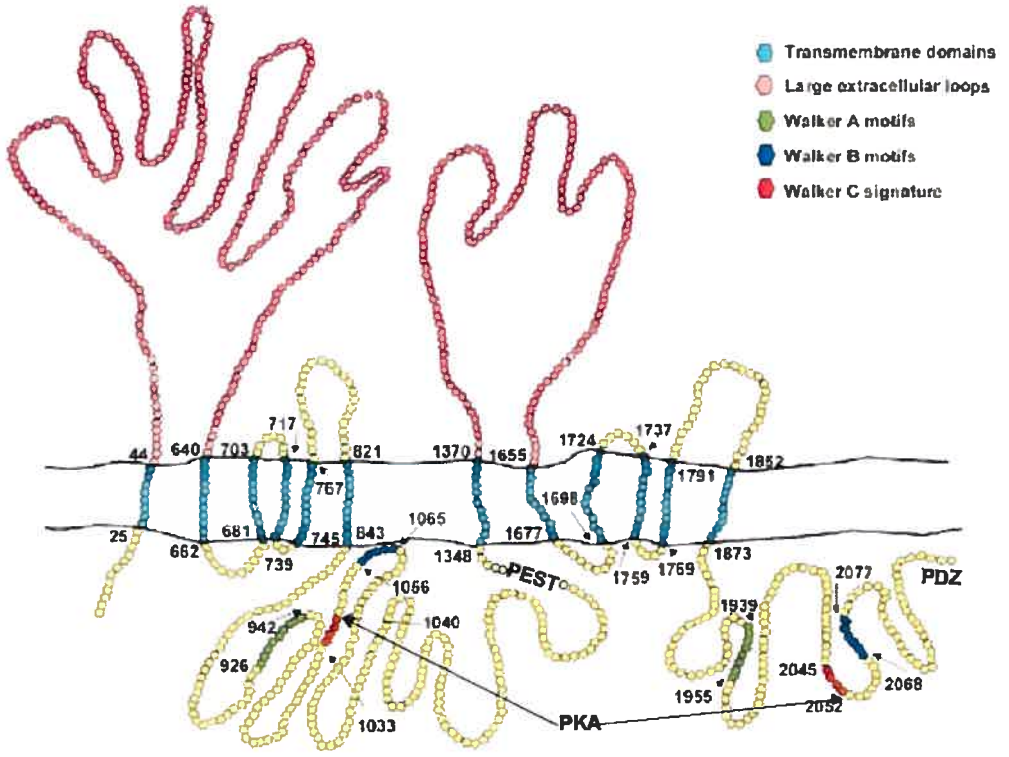

Figure I.14 : Structure du transporteur ABCA1. Deux segments extracellulaires permettent la liaison du ligand et la dimérisation alors que les domaines intracellulaires contiennent, en plus du domaine de liaison de l'ATP, des motifs de régulation post-traductionnelle comme une séquence PEST, deux séquences consensus de phosphorylation par la PKA et un motif PDZ. Adapté de [127]

Domaines extracellulaires

Les domaines extracellulaires seraient le site d'interaction avec les ligands puisque des mutations ponctuelles dans ces domaines diminuent l'association de l'^{125}I-apoA-I, sans pour autant affecter l'expression de l'ABCA1 à la surface membranaire, telle que mesurée par biotinylation de surface [128], [129],[130]. Aussi, sur gel d'acrylamide, l'ABCA1 migre habituellement sous forme de doublet. Ceci pourrait être dû au clivage du peptide signal. Mais une autre possibilité existe : il a été postulé, puis confirmé par traitement de lysats cellulaires à la N-glycosydase F précédant la migration sur gel, que l'ABCA1 est une glycoprotéine [125]. À la suite de leur étude sur l'ABCA4, Bungert et coll. ont suggéré que la glycosylation des ABC stabilisait ces transporteurs et protégerait contre la dégradation [131]. Finalement, nous avons proposé que les cystéines des domaines extracellulaires

pourraient former des ponts disulfures intramoléculaires ou avec un autre monomère d'ABCA1, et ainsi former des dimères (voir chapitre 5 ou référence [93]).

Domaines intracellulaires

Les domaines intracellulaires contiennent, bien entendu, les motifs Walker A, Walker B et «Signature» caractéristiques des ABC. Le rôle des domaines de liaison des nucléotides (NBF) dans l'hydrolyse de l'ATP a été étudié par transfection d'ABCA1 dans des cellules d'insecte Sf9. L'incorporation du 8-azido-(α-^{32}P)ATP dans les NBF d'ABCA1 requiert du Mg^{2+} puisque l'utilisation d'EDTA, un chélateur d'ions divalents, abolit complètement l'incorporation. L'ABCA1 hydrolyse l'ATP lentement, comparativement au transporteur MDR1. Par ailleurs, il semble que ni le cholestérol, ni les phospholipides, ni même l'apoA-I n'active l'hydrolyse d'ATP. Les auteurs de l'étude concluent que l'ABCA1 n'est probablement pas un transporteur actif, mais plutôt un régulateur membranaire, vu son hydrolyse lente de l'ATP [132]. Cette propriété de régulateur plutôt que de transporteur actif a d'ailleurs déjà été attribuée à l'ABCC7 (CFTR).

Motifs structuraux de régulation

D'autres motifs des segments intracellulaires modulent la stabilité, la fonction et la localisation cellulaire de l'ABCA1. C'est le cas de la séquence PEST, des sites consensus de phosphorylation par la PKA et du domaine PDZ.

a) Séquence PEST

Les acides aminés 1283-1306 de l'ABCA1 présentent une séquence appelée « PEST », une séquence riche en proline (P), acide glutamique (E), sérine (S) et thréonine (T). Une séquence PEST est caractéristique d'une dégradation rapide en favorisant habituellement l'ubiquitination, un signal ciblant une protéine pour dégradation protéosomale. La délétion de la séquence PEST de l'ABCA1 quadruple sa quantité, tout en doublant l'association de l'apoA-I, l'efflux de cholestérol et de phospholipides [133]. Toutefois, la séquence PEST influence spécifiquement la dégradation de l'ABCA1 par la protéase calpaïne, tel que démontré par l'utilisation de la calpeptine, un inhibiteur de la calpaïne. La mutagenèse dirigée de deux résidus thréonine (Thr-1286 et Thr-1305) de cette séquence PEST a montré qu'ils sont constitutivement phosphorylés. L'incubation avec l'apoA-I abolit cette phosphorylation. Ceci augmente la demi-vie de l'ABCA1 en inhibant sa dégradation par la calpaïne [134], un phénomène indépendant de l'ubiquitination et qui ne se produit pas si la

séquence PEST est suprimée [133]. L'apoA-I générerait donc une boucle de rétroaction positive en stabilisant son récepteur par déphosphorylation de la séquence PEST.

b) Site consensus de phosphorylation par la protéine kinase A (PKA)

Le traitement de fibroblastes avec du 8-bromo-AMPc augmente l'incorporation de ^{32}P-orthophosphate dans l'ABCA1. Il a été suggéré que cela était dû à une activation de la PKA puisque le composé H-89, un inhibiteur de la PKA, inhibait ce processus [135]. La mutagenèse dirigée a montré que la délétion des sérines 1042 et 2054 de sites consensus de phosphorylation par la PKA ramenait la phosphorylation de l'ABCA1 au niveau basal et réduisait l'efflux de phospholipides de 40% [136]. Finalement, Haidar et coll. ont démontré que l'incubation avec l'apoA-I induisait la phosphorylation d'ABCA1 par une voie dépendante de la PKA [137], entraînant une augmentation concomitante de l'efflux de cholestérol. L'apoA-I joue donc un double rôle en stabilisant l'ABCA1 par déphosphorylation de la séquence PEST et en activant l'efflux de cholestérol par phosphorylation par la PKA.

c) Motif PDZ

Les trois derniers acides aminés de l'ABCA1 forment une séquence PDZ. Le rôle d'un domaine PDZ (site consensus = D/E-T/S-Xϕ, où X = n'importe quel acide aminé et ϕ est un acide aminé hydrophobe, habituellement V, I ou L) est de positionner les protéines les unes par rapport aux autres en les associant avec les composantes membranaires du cytosquelette. EBP50, une protéine adaptatrice, se lie au domaine PDZ de l'ABCC7 (CFTR), le reliant ainsi au cytosquelette [138]. Un système de double hybride et une co-immunoprécipitation a permis d'identifier la β_2-syntrophine comme protéine interagissant avec l'ABCA1. Cette protéine s'associe à l'utrophine, une protéine d'échafaudage sous-membranaire de la famille de la dystrophine. Ceci positionnerait l'ABCA1 dans la membrane hors des radeaux lipidiques (rafts) [139]. Par ailleurs, utilisant une stratégie similaire, l'α_1-syntrophine a aussi été identifiée comme interagissant avec l'ABCA1. D'ailleurs, la délétion de la séquence PDZ abolit cette interaction. Dans un système de co-surexpression de l'α_1-syntrophine et de l'ABCA1, un essai de demi-vie utilisant la cycloheximide pour inhiber la synthèse protéique a indiqué que l'α_1-syntrophine quintuple la stabilité de l'ABCA1. La co-surexpression a aussi révélé que l'efflux de cholestérol

double en présence de l'α_1-syntrophine [140]. Le domaine PDZ est donc essentiel au maintien de la fonction d'ABCA1.

4.4 Interaction ABCA1/apoA-I et efflux

Lipidation de l'apoA-I : 3 théories

La première étape dans la génération des HDL est la lipidation par l'ABCA1 de l'apoA-I pauvrement lipidée. Deux théories s'opposent sur la nature de cette interaction. La première propose une interaction indirecte : l'ABCA1 générerait des microdomaines instables dans la membrane plasmique, permettant l'enchassement de l'apoA-I. L'apoA-I gagnerait des lipides par microsolubilisation, un processus indépendant de l'ABCA1 (figure I.15). Cette suggestion provient de 3 observations : 1) une ABCA1 mutée dans le domaine ATPasique inhibe la liaison de l'apoA-I sans pour autant affecter la présence de l'ABCA1 à la membrane; 2) l'association de l'apoA-I à la membrane, telle que mesurée par FACS (fluorescence par cytométrie en flux), n'est pas parfaitement corrélée à la quantité d'ABCA1-GFP présente à la membrane; et 3) la mobilité latérale de l'ABCA1-GFP dans la membrane est différente de celle de l'apoA-I. Cette théorie suggère aussi que l'apoA-I s'associe à la phosphatidylsérine flippée au feuillet externe par l'ABCA1. Toutefois, Smith et coll. ont clairement montré que l'annexine-V et l'apoA-I ne compétitionnent pas l'un contre l'autre dans leur association à la membrane [141].

Dans la deuxième théorie, mise de l'avant par pontage intermoléculaire (« cross-linking ») [100],[142],[128],[55], l'apoA-I serait un ligand pour l'ABCA1, son récepteur, qui transférerait des lipides sur l'apoA-I. L'enrichissement de l'apoA-I en lipides diminuerait son affinité pour l'ABCA1, ce qui faciliterait la dissociation du ligand. Ce modèle est aussi supporté par le fait que des mutations dans le domaine extracellulaire de l'ABCA1 inhibent la liaison de l'apoA-I, sans pour autant affecter la présence de l'ABCA1 à la membrane [128],[137].

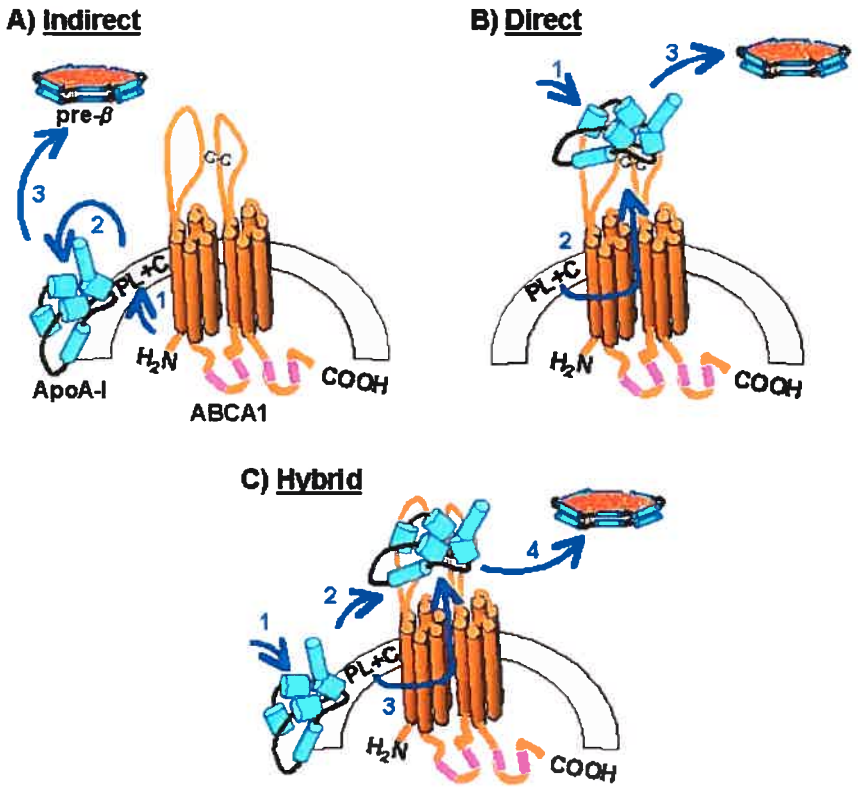

Figure I.15 : Trois modèles de lipidation de l'apoA-I par l'ABCA1. **A)** Le premier modèle propose que l'apoA-I s'accapare des lipides dans des domaines instables de la membrane créés par l'activité phosphatidylsérine translocase de l'ABCA1. **B)** Le deuxième modèle suggère une interaction protéine/protéine directe entre un ligand (apoA-I) et son récepteur (ABCA1). L'ABCA1 transférerait alors des lipides (PL + C) sur l'apoA-I, diminuant ainsi l'affinité entre les deux et provoquant la dissociation. **C)** Un modèle hybride est proposé : l'apoA-I se lie aux domaines riches en phosphatidylsérines générés par l'ABCA1 et diffuse dans la membrane jusqu'à rencontrer l'ABCA1. Ensuite, dans une interaction protéine/protéine, l'ABCA1 transfère de la phosphatidylcholine et du cholestérol sur l'apoA-I, permettant sa dissociation de la membrane.

Il existe aussi un troisième modèle, hybride des deux premiers, qui propose que 1) l'apoA-I s'enchasse dans les microdomaines instables générés par l'ABCA1, 2) l'apoA-I diffuse jusqu'à l'établissement d'un contact protéine-protéine entre l'apoA-I et l'ABCA1 tandis que 3) cette dernière facilite l'efflux en transférant davantage de lipides sur l'apoA-I. 4) L'apoA-

I suffisamment lipidée pourrait alors quitter la membrane sous la forme d'une particule discoïdale pré-β [9]. Les résidus de l'apoA-I responsables de l'interaction avec l'ABCA1 n'ont pas encore été identifiés. Cependant, tel que mentionné précédemment, Natarajan et coll. ont proposé un rôle important de l'hélice 9 de l'apoA-I dans la liaison à l'ABCA1 [66].

Internalisation du complexe

Avant l'identification de l'ABCA1, Smith et coll. avaient montré par microscopie électronique que l'apoA-I peut-être internalisée dans les puits de clathrine de macrophages murins RAW dans un processus dépendant de l'AMPc [143]. Plus récemment, Neufeld et coll. ont montré par microscopie de fluorescence que l'ABCA1-GFP transite entre des compartiments endosomaux et la membrane plasmique [144]. Finalement, utilisant la microscopie confocale avec de l'apoA-I couplée à Alexa568 et l'ABCA1-GFP, le même groupe a démontré qu'à 37 °C l'apoA-I était d'abord internalisée avec l'ABCA1 pour ensuite être resécrétée dans le milieu [145]. Utilisant des macrophages murins, Smith et coll. ont montré qu'à 21 °C, l'interaction apoA-I/ABCA1 démontre au microscope confocal une localisation en surface et aucun efflux de cholestérol n'est détecté. Par contre, dès que les cellules sont mises à 37 °C, une internalisation se produit et l'efflux de cholestérol devient détectable [146]. Les évidences pointent donc en direction d'une possible internalisation du complexe.

Efflux en deux étapes

Le transporteur ABCA1 est reconnu pour son implication dans l'efflux de cholestérol et de phopholipides. Les fibroblastes de patients avec mutation dans l'ABCA1 ont des efflux de cholestérol **et** de phospholipides abaissés [54]. Toutefois, le mécanisme moléculaire d'efflux lipidique par l'ABCA1 reste indéterminé. Fielding et coll. ont proposé un mécanisme en deux étapes après avoir comparé l'efflux de cellules exprimant ou non l'ABCA1. Par exemple, les cellules endothéliales qui n'expriment pas l'ABCA1 sont capables d'efflux de cholestérol seulement si l'apoA-I a été préalablement phospholipidé par incubation avec des fibroblastes exprimant l'ABCA1 [147]. De plus, en utilisant l'acide okadaïque, un inhibiteur de phosphatases reconnu pour inhiber l'action des *caveolae*, l'efflux de cholestérol était inhibé sans pour autant affecter l'efflux de phosphatidylcholine. Ils proposent alors un modèle d'efflux en deux étapes, la première étant l'ajout de phosphatidylcholine sur l'apoA-I, un processus médié par l'ABCA1. Cette phospholipidation de l'apoA-I la rendrait compétente à ensuite accepter du cholestérol

provenant des *caveolae*, un mécanisme indépendant de l'ABCA1. Des résultats similaires furent obtenus par Wang et coll. Dans un système différent, ils ont pré-incubé les cellules avec de la cyclodextrine, afin de dégarnir la membrane de son cholestérol. Un efflux réalisé ensuite révèle, tel qu'attendu, un efflux de cholestérol diminué sans pour autant affecter ni la liaison d'apoA-I ni l'efflux de phospholipides [101]. Cela suggère que les efflux de cholestérol et de phospholipides peuvent être dissociés. Il est alors proposé que l'ABCA1 transfère de la phosphatidylcholine sur l'apoA-I tandis que l'efflux de cholestérol serait secondaire et indépendant de l'ABCA1.

Toutefois, Smith et coll. rapportent qu'ils sont incapables de reproduire cette notion d'efflux en deux étapes dans des macrophages murins RAW. Pour eux, le prétraitement à la cyclodextrine diminue l'efflux de cholestérol et de phospholipides de façon équivalente. Ils suggèrent que le traitement à la cyclodextrine cause des dommages cellulaires et relâche des phospholipides dans le milieu, même en absence d'apoA-I [146]. Honnêtement, nous n'avons jamais pu, nous non plus, distinguer l'efflux de cholestérol de l'efflux de phospholipides (données non-publiées), même dans des temps d'incubation très courts. De plus, il existe une corrélation directe entre ces deux efflux [148]. En conclusion, s'il n'est pas certain que l'ABCA1 transporte spécifiquement du cholestérol, son importance dans la régulation de cet efflux est indiscutable [149]. Par contre, le mécanisme sous-jacent par lequel l'ABCA1 gère cet efflux reste à élucider.

<u>L'ABCA1, récepteur pour plusieurs ligands</u>

Bien que la littérature ait beaucoup focalisé sur l'interaction apoA-I/ABCA1, d'autres apolipoprotéines peuvent interagir avec le transporteur. En effet, Remaley et coll. ont surexprimé l'ABCA1-GFP dans des cellules Hela qu'ils ont incubées avec les apolipoprotéines A-II, A-IV, C-I, C-II, C-III et l'apoE. Ces apolipoprotéines sont dites « échangeables », parce qu'elles peuvent s'associer réversiblement avec les lipoprotéines circulantes. Toutes ces apolipoprotéines ont démontré un efflux de cholestérol et de phospholipides de 2 à 4 fois plus important dans les cellules exprimant l'ABCA1 *versus* les cellules-contrôle. De plus, l'apoA-II a autant d'affinité que l'apoA-I ($K_{d\ apoA\text{-}II}$ = 0.58 µg/ml; $K_{d\ apoA\text{-}I}$ = 0.60 µg/ml) pour l'ABCA1 et peut compétitionner pour l'association à l'ABCA1. C'est la présence d'hélices amphipatiques sur ces apolipoprotéines qui expliquerait leur capacité d'interagir avec l'ABCA1 et de faire l'efflux de lipides [150]. Par ailleurs, nous avons aussi étudié la capacité de l'apoE à interagir avec l'ABCA1. Un essai

de compétition de liaison a révélé que l'apoE est un meilleur compétiteur que les HDL_3 dans la liaison d'^{125}I-apoA-I/ABCA1. De plus, l'interaction apoE/ABCA1 génère des particules pré-βLpE, ainsi qu'un efflux de cholestérol et de phospholipides dans une cinétique similaire à celle de l'apoA-I [151]. Finalement, dans le but thérapeutique d'augmenter les niveaux de HDL circulants, Remaley et coll. ont testé l'habileté de peptides amphipatiques synthétiques à faire l'efflux les lipides de cellules Hela surexprimant l'ABCA1. Ces peptides synthétiques, mimant l'action de l'apoA-I, augmentent la phosphorylation de l'ABCA1, le stabilisent [152] et effluent du cholestérol et des phospholipides [153]. L'ABCA1 est donc une protéine aux multiples partenaires afin de débarrasser les cellules de leur surplus en lipides.

4.5 Autres fonctions

L'ABCA1 est un transporteur à plusieurs visages. D'autres rôles que l'efflux de lipides par l'apoA-I ont été proposés pour l'ABCA1 : réarrangement du cytosquelette [154], [142], [155] et endocytose [156], production d'apoE par les macrophages [157] et d'apoA-I par le foie [28], efflux d'α-tocophérol (vitamine E) [158], etc. Toutefois, cela déborde du contexte du présent travail ou n'est pas prérequis à la pleine compréhension de cette thèse.

I.5 Conclusion

Le métabolisme des lipoprotéines assure l'homéostasie du cholestérol dans l'organisme. Les HDL jouent un rôle particulièrement important dans le transport à rebours du cholestérol. Son apolipoprotéine la plus importante, l'apoA-I est une partenaire de l'ABCA1 pour former les particules HDL naissantes. La compréhension du mécanisme par lequel l'ABCA1 transfère des lipides sur l'apoA-I pour former les HDL est crucial à l'établissement de stratégies potentielles pour prévenir les maladies cardiaques. C'est donc dans cette optique que le travail de cette thèse a été entrepris avec l'étude de la régulation transcriptionnelle d'ABCA1, son interaction avec l'apoA-I, et la structure quaternaire qui lui est requise pour effectuer sa fonction.

Chapitre II

Énoncé du projet

II.1 Introduction

Les facteurs de risques pour l'apparition de maladies cardiovasculaires sont bien connus. Parmi eux se trouvent des niveaux élevés de LDL-cholestérol et des niveaux bas de HDL-cholestérol.

Les patients atteints de la maladie de Tangier présentent des niveaux de HDL très bas (presque absents) ainsi que des problèmes cardiovasculaires précoces. Notre laboratoire s'intéresse depuis les dix dernières années à un phénotype de sévérité intermédiaire, mais semblable à celui des patients de Tangier : la déficience familiale en HDL (FHD). Des études d'efflux de cholestérol réalisées à l'aide de fibroblastes cutanés de ces patients ont révélé que le processus de lipidation de l'apolipoprotéine A-I, la composante protéique majeure des HDL, y était défectueux.

Récemment, le gène encodant le transporteur « ATP-binding cassette A1 » (ABCA1) a été identifié comme responsable de la maladie de FHD/Tangier. Les transporteurs ABC utilisent l'hydrolyse d'ATP comme source d'énergie pour effectuer le transport actif de molécules variées. Il est attendu que l'ABCA1 transporte du cholestérol vers la membrane externe et facilite l'efflux de lipides vers l'apoA-I formant ainsi des particules HDL naissantes. Ceci initie le transport à rebours du cholestérol (« reverse cholesterol transport »), un processus défectueux chez les patients avec la maladie de FHD/Tangier.

II.2 Sélection du modèle d'étude

Depuis plusieurs années, notre laboratoire utilise des fibroblastes humains obtenus par biopsie cutanée des patients. Ce modèle comporte plusieurs avantages.

Accessibilité et entretien ex vivo

Premièrement, il s'agit d'un type cellulaire facile à obtenir. La biopsie cutanée est une opération non invasive ne laissant qu'une cicatrice presque invisible. De plus, la culture *ex vivo* des fibroblastes dans un pétri est une chose relativement aisée. Enfin, ces cellules peuvent être congelées et conservées pour une longue durée. Elles comportent par contre un désavantage: elles sont très difficilement transfectables, ce qui rend toute modification génique difficile.

Homéostasie cellulaire

Deuxièmement, les fibroblastes représentent un type cellulaire dans lequel le contrôle de l'homéostasie du cholestérol est relativement simple. Le cholestérol peut provenir des LDL captés et endocytés par leur récepteur de la surface membranaire. Il peut aussi provenir de la biosynthèse *de novo* ou être stocké sous forme estérifiée dans le réticulum endoplasmique. Finalement, le surplus de cholestérol peut être rejeté à l'extérieur (efflux) de la cellule vers un accepteur habituellement plasmatique comme l'apoA-I. En comparaison, le macrophage possède, en plus des voies présentes chez le fibroblaste, des récepteurs de lipoprotéines oxydées et le récepteur SR-BI, capable lui aussi d'interagir avec les HDL et l'apoA-I. Finalement, même si l'hépatocyte est au centre du contrôle métabolique des niveaux de cholestérol, il possède deux voies additionnelles pour se débarrasser de son cholestérol : la transformation/sécrétion sous forme d'acides biliaires, et la production de VLDL. Ceci compliquerait énormément l'étude de l'interaction ABCA1/apoA-I dans ce type cellulaire.

II-3 Questions posées

Puisque la fonction attendue de l'ABCA1 est de faciliter l'efflux de cholestérol, il a été logique d'observer qu'un surplus de cholestérol cellulaire augmente la transcription du gène. Cette transcription est sous le contrôle d'un promoteur principal ainsi que de trois promoteurs alternatifs situés dans l'intron 1. Chacun des promoteurs possède des éléments DR4 permettant la liaison du facteur de transcription « liver-X-receptor » (LXR) activé par les hydroxystérols. Ce facteur s'associe au « retinoid-X-receptor » (RXR) activé par l'acide rétinoïque. Par ailleurs, il a été rapporté que l'AMPcyclique augmentait la trancription du gène dans les macrophages murins. Afin de déterminer les paramètres expérimentaux permettant d'utiliser notre modèle cellulaire pour étudier les mécanismes d'efflux de cholestérol, nous avons posé la question :

1) **Par quel(s) mécanisme(s) le cholestérol cellulaire module-t-il la transcription d'ABCA1?**

La lipidation de l'apoA-I par l'ABCA1 suppose une interaction entre les deux molécules. Deux modèles sont proposés pour expliquer cette interaction. Dans le premier, aucune interaction physique entre les deux molécules n'est requise et l'apoA-I devient lipidée par contact avec les domaines lipidiques générés par l'ABCA1. Dans le deuxième modèle, l'ABCA1 transfère activement des lipides sur l'apoA-I pendant une interaction directe protéine/protéine. Or, avant la découverte reliant l'ABCA1 à la maladie de Tangier, un vieux modèle de genèse et de maturation des HDL proposait que les premiers accepteurs de lipides sont les particules pré-β discoïdales pauvrement lipidées. Il est donc attendu que le résultat de l'interaction apoA-I/ABCA1 soit une particule migrant en position pré-β. Afin de déterminer la nature de l'interaction entre l'apoA-I et l'ABCA1 et de caractériser le produit de cette interaction, nous avons posé la question :

2) Quel type d'interaction relie l'apoA-I à l'ABCA1, et quelle forme prend le produit de cette interaction?

Il a été proposé que les particules HDL possèdent une, deux, trois et même quatre molécules d'apoA-I, selon leur degré de maturation. Cependant, le mécanisme *moléculaire* d'interaction entre l'apoA-I et l'ABCA1 reste inconnu. Même si l'on sait que d'autres transporteurs ABC peuvent dimériser, aucun modèle existant ne permet de bien expliquer la complexité des particules résultantes de l'interaction apoA-I/ABCA1. Afin de déterminer les pré-requis structuraux d'ABCA1 pour former les HDL naissantes et de caractériser ces particules, nous avons posé la question :

3) Quelle est la structure fonctionnelle de l'ABCA1, et quelle répercussion stochïométrique entraîne-t-elle sur la formation des HDL naissantes?

C'est à ces trois questions que les chapitres suivants tentent successivement de répondre. Toutefois, comme il est impossible de répondre parfaitement à chaque question, et que des réponses incomplètes soulèvent toujours de nouvelles questions, le sixième chapitre proposera un sommaire des résultats obtenus et discutera des nouveaux intérêts suscités.

Chapitre III

Article #1 *

1. Contexte de travail

Historiquement, ce travail fut entrepris peu après la découverte de l'implication de l'ABCA1 dans la maladie de Tangier. À ce moment, très peu d'informations étaient disponibles quant à la régulation transcriptionnelle d'ABCA1. Langmann et coll. [108] avaient démontré que la transcription d'ABCA1 est dépendante de la quantité de stérols cellulaires. Peu après, le promoteur a été cloné et un élément DR4 y fut identifié, suggérant une régulation par les hydroxystérols. Par ailleurs, Oram et coll. [100] ont rapporté que l'ABCA1 était induit dans les macrophages murins par l'AMPc.

Afin d'établir un système permettant d'étudier spécifiquement la fonction d'ABCA1 (voir chapitres IV et V), il était primordial de déterminer le mécanisme de régulation du transporteur.

C'est dans ***le but d'étudier la régulation transcriptionnelle d'ABCA1*** dans notre tissu d'étude (les fibroblastes cutanés humains) que le travail a été entrepris.

Dans l'article suivant,

Maxime Denis a fait la majeure partie du travail et a écrit le manuscrit
Rachel Bissonnette a partagé ses données sur les HepG2 pour réaliser la figure 1
Bassam Haidar a fait l'immunoblot anti-HSP70 de la figure 3D
Larbi Krimbou a donné des conseils pour l'écriture du manuscrit
Michel Bouvier a participé à la supervision
Jacques Genest a supervisé le travail

*** Note de l'auteur :** Cet article mis en page sous forme "pdf" est disponible en annexe

2. Article:

"Expression, Regulation and Activity of ABCA1 in Human Cell Lines"

(Published in *Molecular Genetics and Metabolism* 78 (2003) pp.265-274)

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Running title: Denis *et al.* ABCA1 regulation by sterols in fibroblasts

Word count: Abstract: 192 words
Body: 3803 words (excluding legends and references)
Figure count: 6

Abstract

Mutations in the ATP-binding cassette transporter A1 (ABCA1) gene cause familial high-density lipoprotein (HDL) deficiency (FHD) and Tangier disease (TD). ABCA1 plays a crucial role in active apolipoprotein A-I (apoA-I) lipidation, a key step in reverse cholesterol transport. We compared ABCA1 transcriptional regulation and cholesterol efflux in human skin fibroblasts, monocyte-derived macrophages and hepatocytes (HepG2). 8-Br-cAMP did not increase ABCA1 transcription in these tissues compared to mouse macrophages. We found that ABCA1 is differentially regulated among tissues. While transcription in HepG2 appears to be constitutive, sterols stimulate ABCA1 transcription in fibroblasts and monocyte-derived macrophages. ApoA-I promoted cholesterol efflux in fibroblasts, macrophages and HepG2. Cholesterol homeostasis in fibroblasts is tightly regulated, and ABCA1 mRNA closely follows the cellular mass of free cholesterol (dose- and time-dependant manner). To further determine the mechanism used by fibroblasts to maintain sterol balance, we used a competitive inhibition approach with geranylgeranyl pyrophosphate (GGPP) to block the LXR induction pathway. GGPP blocked basal, 22-(R)-hydroxycholesterol- and cholesterol-induced ABCA1 expression. Taken together, these results demonstrate that : 1) ABCA1 expression varies among tissues, and 2) cholesterol conversion to hydroxycholesterol is an important mechanism for the maintenance of cholesterol homeostasis in fibroblasts.

Keywords: ABCA1, cholesterol, hydroxysterol, gene regulation, fibroblast

Abbreviations:

ABCA1: ATP-binding cassette transporter A1

BSA: Bovine serum albumine

BAC: Bacterial artificial chromosome

TD: Tangier disease

FHD: familial HDL deficiency

HDL: high density lipoprotein

LDL: low density lipoprotein

FBS: fetal bovine serum

LPDS: lipoprotein deficient serum

cAMP: cyclic adenosine monophosphate

LXR: liver X receptor

RXR: retinoic X receptor

9CRA: 9-*cis* retinoic acid

22OH: 22(R)-hydroxycholesterol

HSF: human skin fibroblasts

FC: free cholesterol

CE: cholesteryl ester

ApoA-I: apolipoprotein A1

DR: direct repeat

LDL-C: low density lipoprotein-cholesterol

HDL-C: high density lipoprotein-cholesterol

PMSF: phenylmethylsulfonide fluoride

PVDF: polyvinylidene fluoride

SDS-PAGGE: sodium dodecylsulfate-polyacrylamide gradient gel electrophoresis

Introduction

The ABCA1 gene codes for the ATP-binding cassette transporter A1 required for the efflux of phospholipids and cholesterol from cells. Heterozygous patients for mutations at the ABCA1 gene locus cause familial HDL deficiency (FHD) whereas the homozygous or compound heterozygous forms cause Tangier disease [1-5]. The ABCA1 protein is thought to promote active transport of phospholipids and cholesterol to the plasma membrane where they become available for efflux onto acceptor particles [6]. The main physiological acceptors for efflux are lipid poor apoA-I particles, the precursor of HDL. Absence of functional ABCA1 leads to a marked reduction in apoA-I-mediated cellular cholesterol efflux and the lack of formation of mature, spherical HDL [7]. Immature particles are then rapidly catabolized, most likely in the kidney or liver, causing low HDL levels [6]. Both the homozygous and heterozygous forms are associated with an increased risk of coronary artery disease [8-9].

The ABCA1 transporter is predicted to have 12 transmembrane domains and to be synthesized with a signal peptide [10-11]. As in other ABC transporters, two intracellular segments of the protein contain nucleotide-binding domains (NBDs) that allow the protein to bind and slowly hydrolyse ATP [12]. The extracellular portion is glycosylated [11] and the transporter appears to shuttle between late endosomal compartments and the plasma membrane [13] where ABCA1 is thought to directly [14-16] or indirectly [17] interact with apoA-I. Studies on ABCA1 gene regulation performed in mouse or human macrophages have revealed that cAMP analogs [14-15,18], modified LDL [19], or hydroxycholesterol [20-22] induce ABCA1 transcription. The regulation by hydroxysterols is explained by the presence of functional DR4 elements in the promoter and in intron 1 [23] of the ABCA1 gene. These elements allow the binding of a heterodimer composed of the liver-X-receptor (LXR) and the retinoid-X-receptor (RXR) and enhance the transcription of the gene. Although the gene is expressed in most cell types, many studies on the regulation of its expression have concentrated on mouse macrophages. This is probably due to the observation that cholesterol-laden macrophages were found to accumulate in lymphoid tissues from Tangier disease patients [6]. However, a recent report suggests that macrophage-specific ABCA1 does not contribute significantly to plasma HDL levels [24]. This finding suggests that ABCA1-mediated lipid efflux from other tissues contributes to HDL-cholesterol levels.

For many years, human skin fibroblasts (HSF) have been used as a model to study cellular cholesterol homeostasis and, more recently, cellular cholesterol efflux. Cholesterol homeostasis is maintained in fibroblasts by four mechanisms: 1) cholesterol influx by the LDL-receptor pathway, 2) *de novo* synthesis by the HMG-CoA reductase pathway; 3) equilibrium between esterified and free cholesterol by the acyl-CoA cholesterol acetyltransferase (ACAT); and 4) ABCA1-mediated cholesterol efflux [25]. Unlike fibroblasts, macrophages can become foam cells. This is partly due to the expression of scavenger receptors for modified lipoproteins that are not transcriptionally regulated by sterols [26]. This suggests that cellular lipid metabolism is regulated differently in that cell type. Indeed, fibroblasts do not produce large amounts of oxysterols, as is the case in macrophages, hepatocytes and adrenal cells. Moreover, oxysterols are toxic to some fibroblast and endothelial cell lines [27-28].

Based on these considerations, we studied the *ex vivo* regulation of endogenous ABCA1 in human cell lines and focused on human skin fibroblasts. We found that the conversion of cholesterol to hydroxycholesterol is an important mechanism to regulate sterol homeostasis in that cell type.

Methods

Materials: All reagents for cell culture were from Gibco (Invitrogen), and all the others were from Sigma. Avasimibe (CI-1011) was a generous gift of Pfizer. Hydroxysterols and cholesterol were dissolved in ethanol at a concentration of 10 mg/ml. 8-Br-cAMP was dissolved in water at a concentration of 0.23 M. HSP70 antibody was from Transduction Lab, Lexington, KY.

Cell culture: Primary cultures of HSF obtained from punch biopsy were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, non-essential amino acids, and 10% fetal bovine serum (FBS). J774 cells were grown in RPMI1640 medium containing glutamine to which were added 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS. HepG2 cells (ATCC) were grown in DMEM-F-12, pH 7.15, penicillin (100 U/ml) and streptomycin (100 mg/ml) and 10% fetal bovine serum (FBS). Trypsin (0.25%), 0.03% EDTA solution was used to separate the cells and 500, 000 cells were seeded in 60 mm plates coated with 6 $\mu g/cm^2$ rat tail collagen type I, as described by Dixon et al [29]. All inductions were performed in medium + BSA 2 mg/ml without serum for 24 h.

Macrophage isolation

Monocyte-derived macrophages from a control subject were isolated on Ficoll-Paque gradient (Amersham/Pharmacia), following the manufacturer's protocol. Briefly, 136ml of blood was mixed with an equal volume of a balanced salt solution (NaCl 126 mM, 0.01 % D-glucose, $CaCl_2$ 5 µM, $MgCl_2$ 98 µM, KCl 0.54 mM, Tris 14.5 mM, pH = 7.6) and layered on top of Ficoll-Paque gradient. Following centrifugation (400 g for 30 min), the white layer corresponding to leukocytes was extracted, washed three times in balanced salt solution, once in RPMI medium and the cells were seeded in 60mm petri dishes (15 million cells/dish) in RPMI medium containing 10% autologous serum. Monocytes were allowed to attach to the bottom of the dish for 16h. At that time, a wash with culture medium removed non-adherent lymphocytes. Monocytes were treated for 7 days with macrophage-colony stimulating factor (M-CSF) (10 ng/ml) (Peprotech, CA) and controlled for differentiation into macrophages by testing for the CD68 antigen expression by Western blot (antibody from Dako, Mississauga, Ontario, Canada).

Serum and lipoprotein preparation: LPDS was prepared by ultracentrifugation of FBS using previously described methods [30]. Low density lipoprotein (LDL) and high density lipoprotein subfraction 3 (HDL_3) were obtained by potassium bromide density gradient ultracentrifugation by a previously described protocol [30] on human serum collected from healthy donors.

Cholesterol efflux: Performed as described [1]. Briefly, half-confluent cells were labeled with ^{3}H-Cholesterol (0.2 µCi/ml) and grown until confluence. Cells were then washed 5X with PBS/BSA and loaded with free cholesterol (20 µg/ml) for 24h in DMEM/BSA 2 mg/ml. Cells were washed 2X with PBS/BSA and allowed to equilibrate for 24h in DMEM/BSA 1 mg/ml. A 24 h efflux was performed by changing medium to DMEM/BSA 1 mg/ml containing (or not) 10 µg/ml delipidated apoA-I. The efflux medium was recovered, cells were lysed in NaOH 0.1N and aliquots from the medium and cells were counted for β-scintillation. The percentage of efflux was determined as the percent counts in medium over counts in medium + cells.

Cellular cholesterol mass measurements: Performed as described [31], with minor modifications. Briefly, cells were washed two times in ice-cold PBS/BSA 1 mg/ml, twice in ice-cold PBS, and lipids were extracted for 30 minutes at room temperature in 5 ml of hexane:isopropanol (3:2 v/v). The extraction was repeated in 3 ml and the extraction media were combined and evaporated in 12 x 75 mm borosilicate glass tubes under nitrogen. The extraction medium contained 25 µg of stigmasterol and 17.5 µg of stigmasteryl oleate to serve as internal standards for the gas chromatography step. Lipids were resuspended in chloroform and separated by thin-layer chromatography (Analtech, Silica Gel G) in an elution system constituted of heptane:ethyl ether:methanol:acetic acid (80:30:3:1.5). Spots corresponding to sterol and sterol esters were scraped and treated separately. Sterols were extracted from the powder by Folch (chloroform:methanol, 2:1) extraction. Steryl esters were hydrolysed by treatment in 0.5 ml of KOH 0.5 M in methanol for 30 min at 80°C. The released sterols were recovered from the organic phase of an extraction in 1 ml hexane + 0.5 ml water. Both free sterols and steryl-ester-derived sterols were resuspended in chloroform and derivatized for 15 min at 80°C, prior to loading on a gas chromatography column (Hewlett Packard). Cholesteryl mass for each spot was corrected by dividing by protein mass for each sample. All experiments were performed in triplicate.

Probes and Northern blots: A 517 bp probe for human ABCA1 and for mouse ABCA1 were prepared by reverse transcription performed on total RNA obtained from human skin fibroblasts and from J774 mouse macrophages. This was followed by a PCR step using the forward primer 5'- CCT TGG GTT CAG GGG ATT AT-3' and the reverse primer 5'- AGG ATT GGC TTC TTC AGG ATG TCC-3'. The amplified fragment were subcloned into pGEM-T (Promega) and used to transform JM109 cells and sequenced to ensure the proper identity. Probes were prepared by digestion with Sal I and Sac II and the insert was excised out of the agarose gel and ^{32}P-labelled using the Amersham/Pharmacia Oligolabeling kit and used as a probe at a concentration of 10^6cpm/ml in Northern blots. A glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe was obtained using a similar strategy. Ten to fifteen micrograms/lane of total RNA extracted from cells using the RNEasy kit (Quiagen) were loaded on a 1% formaldehyde agarose gel. RNA was transferred to a Hybond N+ (Amersham) membrane and probed. Bands were quantified on a Storm phosphorimager (Molecular Dynamics). To correct for loading, the signal of the bands was divided by the signal for the 18S ribosomal subunit, and the control condition was adjusted to 100%.

mRNA half life:

Ninety percent confluent HSF were grown for 24 h in DMEM containing 5% LPDS. The medium was changed to DMEM/BSA 2 mg/ml containing 20 µg/ml free cholesterol or vehicle alone (ethanol) for 24 h. The medium was then changed to DMEM containing the transcription inihibitor DRB (5,6-dichlorobenzimidazole riboside) (Sigma) [32] at a concentration of 10 µg/ml and incubated for the indicated times. The medium was removed and RNA extraction was performed using the RNEasy kit (Quiagen), prior to Northern blotting on 15 µg of total RNA. Bands were quantified on a Storm phosphorimager (Molecular Dynamics) and expressed as a ratio of the signal divided by the 18S ribosomal subunit, with the control condition adjusted to 100%. The interpolation and calculations of half-life values were performed using the GraphPad Prism 3.02 software (GraphPad Softwares Inc.)

ABCA1 antibody generation: A peptide encoding the amino acids 867-GEEDEKSHP GSNQKRISE-885 of the human ATP-binding cassette-1 derived from the published sequence (Accession # O95477) was synthesized at the McGill University Sheldon

Biotechnology Center (Montreal, PQ, Canada), according to the multiple antigenic peptide (MAP) method of Tam [33]. New Zealand White rabbits were immunized with the peptide. The antibody was purified on a Protein A-Sepharose coupled bead (Amersham /Pharmacia Biotech) column and eluted with glycine 0.1 M pH 2.5. Fractions containing the antibody were pooled, dialyzed and concentrated by centrifugation in a Centriplus 10 column (Amicon). The concentration of antibody was adjusted to 2.5 mg/ml in a mixture containing glycerol 50%, NaCl 150 mM and BSA 1 mg/ml and stored at -20°C until further use.

Immunoblotting: The cells were cholesterol loaded in DMEM/BSA 2 mg/ml supplemented with 20 µg/ml of free cholesterol for the indicated times. The cells were washed twice in ice-cold PBS/BSA, twice in ice-cold PBS and scraped in lysis buffer (20 mM Tris-HCl, 0.32 M sucrose, pH 7.4, 50 mM 2-mercaptoethanol, 0.2 mM PMSF, 20 µg/ml leupeptin, 25 µg/ml aprotinin) and homogenized with a 2 ml tight-fitting dounce homogenizer. The homogenate was cleared of cell debris by gentle centrifugation (1000 g for 10 min at 4°C). The post-nuclear supernatant (PNS) was removed, stored on ice and an aliquot was used for protein determination with Bradford reagent (Bio-Rad) according to the manufacturer's instructions. Ten micrograms of PNS proteins were migrated on a 4-12.5% SDS-PAGGE and transferred to a PVDF-ImmobilonP membrane (Millipore). The membrane was blocked and incubated with the purified anti-ABCA1 "DEN-3" antibody diluted 1:1000 in tris-buffered saline containing Tween (TBS-T) +1% dehydrated for 90 min. The membrane was incubated for 90 min in TBS-T + 1% dry milk + 1:12500 horseradish peroxidase-coupled rabbit secondary antibody (Pharmacia). Immunoreactive bands were revealed by chemiluminescence with the "Supersignal" reagent obtained from Pierce. The membrane was exposed for 1 min to a Kodak Xomat film.

Results

ABCA1 is differentially regulated in tissues

Recent studies have shown that macrophage ABCA1 is regulated at the transcriptional level by many agents such as AcLDL [19], cAMP analogs [14-15,18] and by the LXR / RXR agonists hydroxysterols and 9-*cis*-retinoic acid [20-22]. To determine whether ABCA1 is regulated in similar or different manners in all tissues, the effect of cholesterol (10 µg/ml), 8-Br-cAMP (0.3 mM), and 22(R)-hydroxycholesterol (2.5 µg/ml) plus 9-*cis*-retinoic acid (10 µM) for 24 h was tested by Northern blot analysis in HSF, human monocyte-derived macrophages and human hepatocytes (HepG2). As shown in figure 1, cholesterol and 22OH together with 9CRA were strong modulators of ABCA1 mRNA in fibroblasts, but not necessarily in other cell types. In contrast to HSF, cholesterol alone was not a potent modulator of ABCA1 mRNA in macrophages or HepG2. Also, 8-Br-cAMP was a strong modulator of ABCA1 mRNA expression in J774 murine macrophages whereas it had little or no effect on ABCA1 mRNA levels in human tissues, strongly arguing for a differential regulation between cell types and species. Figure 2 also shows

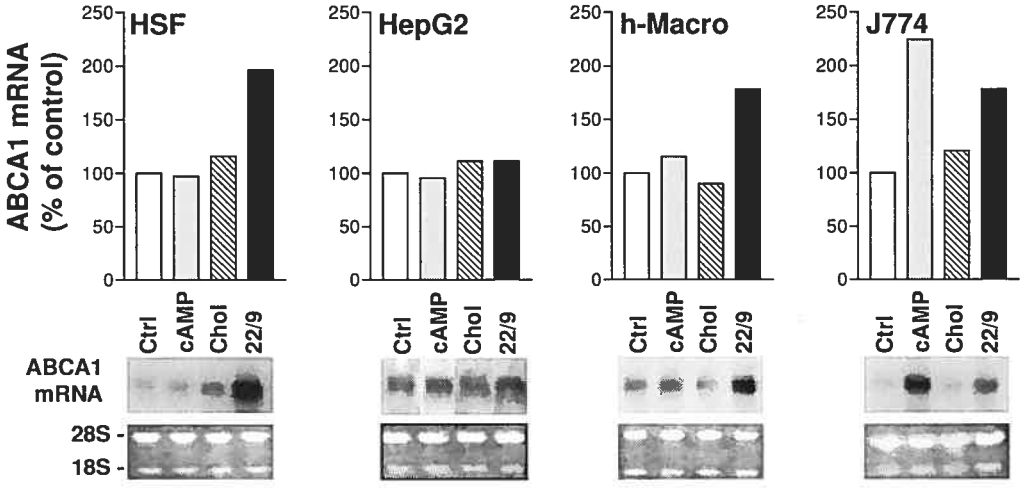

Figure 1. Differential regulation of ABCA1 in human and mouse tissues. Human skin fibroblasts (HSF), HepG2 cells (HepG2), human monocyte-derived macrophages (h-Macro) and mouse macrophages J774 cells (J774) were incubated for 24 h in medium/BSA 2 mg/ml containing either vehicle (ctrl), cholesterol 10 µg/ml, (chol), 8-bromo-cyclic adenosine monophosphate (cAMP) 0.3 mM, 22(R)-hydroxycholesterol 2.5 µg/ml + 9 cis-retinoic acid 10 µM (22/9). Total RNA was extracted and a Northern blot was performed using an anti-human or anti-mouse ABCA1 probe. Blots were quantified and results are expressed as a percentage of the control condition for each cell line. 28S and 18S ribosomal subunits were used to control for loading. The blot shown is representative of two different experiments.

that ABCA1 function also correlates with mRNA levels in most tissues, with the conclusion that whenever ABCA1 is present and modulated, an apoA-I-specific cholesterol efflux can occur.

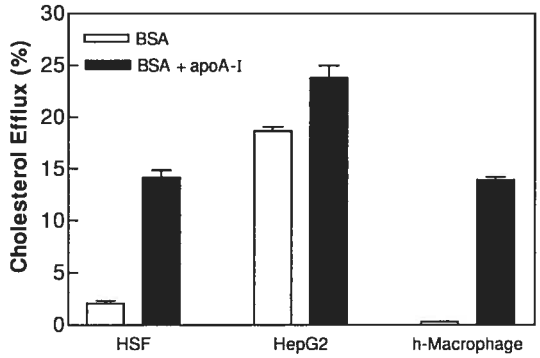

Figure 2. Cholesterol efflux from human tissues. Human skin fibroblasts, HepG2 cells and human monocyte-derived macrophages were loaded for 24h with cholesterol (20 µg/ml) in DMEM/BSA 2 mg/ml, and allowed to equilibrate for 24 h in DMEM/BSA 1 mg/ml. Then, medium was changed to efflux medium (DMEM/BSA 1 mg/ml +/- apoA-I 10 µg/ml) and incubated for 24 h. Percentage efflux represents percent of counts in medium over counts in medium + cells. Results are expressed as mean and standard deviation from an experiment performed in triplicate.

Cellular cholesterol levels modulate ABCA1 mRNA expression.

Recent studies reported that macrophages contribute only mildly to HDL levels, we decided to concentrate on fibroblasts, a cellular model extensively used to study cholesterol efflux and Tangier Disease. With the rationale of ABCA1 being important for cholesterol homeostasis in fibroblasts, we examined the regulation of ABCA1 mRNA levels in cholesterol-loaded cells. To track changes in cholesteryl content, cellular cholesterol and cholesteryl ester mass were determined by gas chromatography. Cholesterol loading resulted in dose- (Fig 3A) and time-dependant (Fig 3B) increases of the cellular content of free and esterified (data not shown) cholesterol, quickly followed by similar increases in ABCA1 mRNA (Fig 3C) and protein (Fig 3D) (Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and Heat-shock protein 70 (HSP70) were used as loading controls). These data suggest a close relationship between cellular cholesterol level and ABCA1 transcription. To rule out a regulatory effect of cholesteryl esters on ABCA1 transcription, the esterification of cholesterol was blocked with avasimibe (Pfizer compound CI-1011), an inhibitor of acyl-cholesterol acetyl-transferase (ACAT) [34]. A dose of 10µM Avasimibe abolished completely the formation of cholesteryl ester with no effect on ABCA1 mRNA levels (data not shown), thus eliminating the possibility of a regulatory effect from esterified cholesterol on ABCA1 transcription.

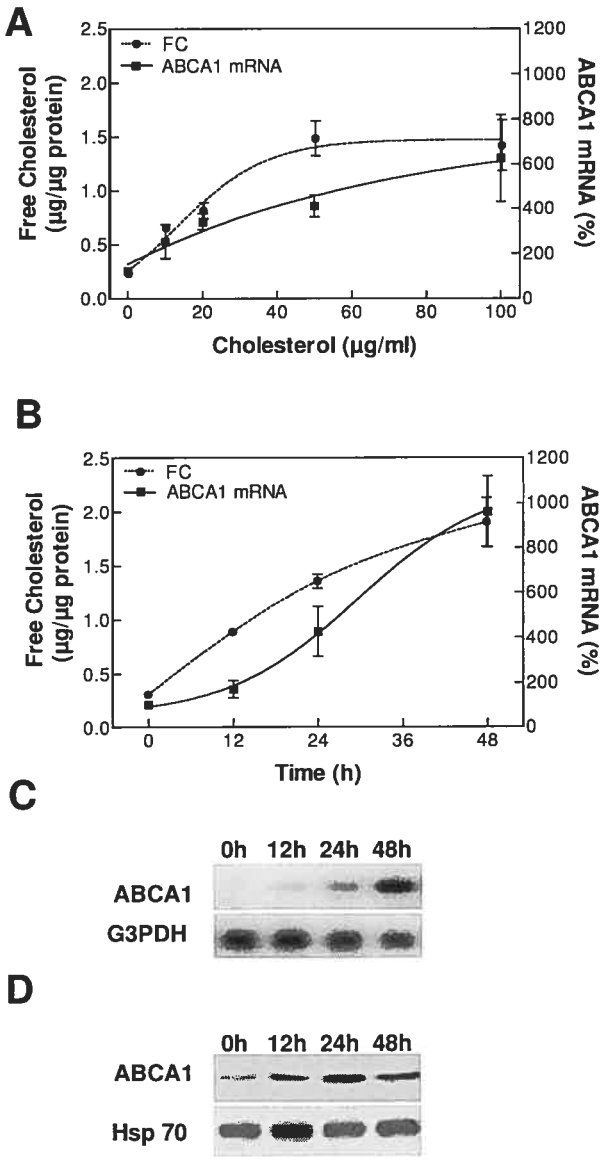

Figure 3. Free cholesterol modulate ABCA1 mRNA in a dose and time-dependent manner. Cellular free cholesterol (FC) (dark circles) was quantified in triplicate by gas chromatography (results expressed as mean +/- standard deviation). ABCA1 mRNA (dark squares) was quantified from two different blots. **A)** HSF were incubated for 24 h in medium containing increasing doses of FC (0-100 µg/ml) or **B)** in medium containing 20 µg/ml FC for increasing periods of time (0-48 h). Results are expressed as a percentage of the control condition. **C)** Northern blot of ABCA1 mRNA controled with G3PDH and **D)** Western blot of ABCA1 protein controled with HSP70.

Cholesterol efflux downregulates ABCA1

We tested the ability of HDL_3 and apoA-I to modulate ABCA1 expression. Figure 4 shows that efflux with HDL_3 or apoA-I (not shown) from LDL-loaded fibroblasts (Fig. 4A) decreased the abundance of the transcript by ~ 60% (Fig. 4B) (~45% for apoA-1, not shown). This suggests a mechanism of retroinhibition for the expression of the gene.

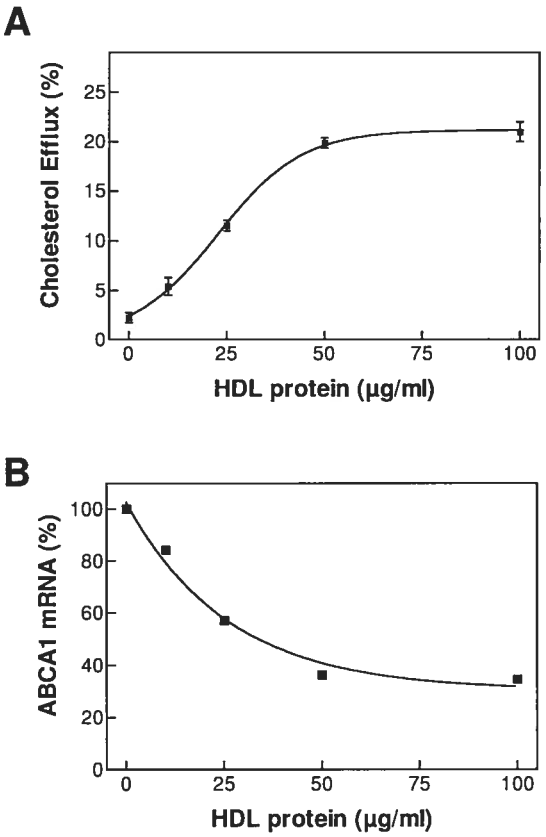

Figure 4. Cholesterol efflux causes retroinhibition of ABCA1 transcription. LDL-loaded fibroblasts (100 µg/ml) were incubated for 24h in DMEM/BSA containing increasing concentrations of HDL_3. A) Cholesterol efflux was measured, or B) total RNA was extracted and 10 µg/lane was used in a Northern blot. ABCA1 mRNA was quantified using a phosphorimager and corrected for loading with the 18S ribosomal subunit. Results are expressed as % of the control condition and are representative of two different experiments.

Cholesterol loading does not alter ABCA1 mRNA half-life

To determine whether cholesterol loading increases ABCA1 mRNA levels by increasing stability of the transcript, mRNA half-life was examined. Human skin fibroblasts were either loaded with 20 µg/ml of free cholesterol or with vehicle alone (ethanol) for 24 h. Following loading, cells were treated with DRB, an inhibitor of RNA transcription, for 0-12

h [32]. Analysis of total RNA by Northern blot revealed no significant difference between the two decay curves (Fig. 5) ($t_{1/2}$ -chol =1.8 h; $t_{1/2}$ + chol = 2.1 h), suggesting that cholesterol loading does not alter mRNA stability and therefore the regulation is likely to be at the transcriptional level.

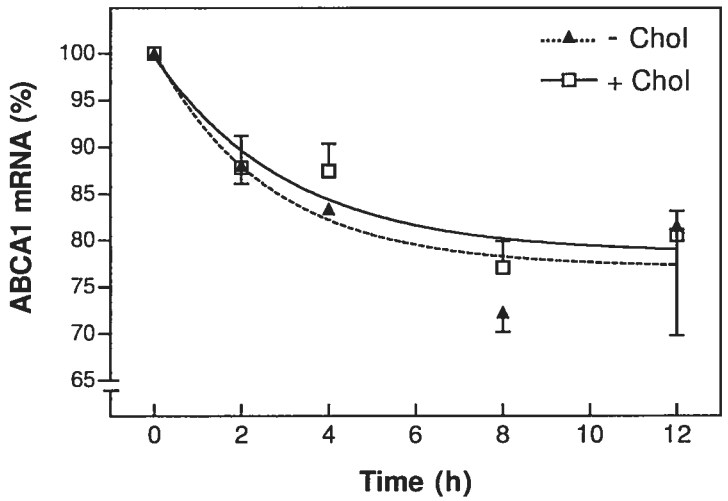

Figure 5. Cholesterol loading does not alter ABCA1 mRNA half-life. Confluent HSF were incubated for 24 h in DMEM/BSA 2 mg/ml containing cholesterol (20 µg/ml) (open squares) or vehicle alone (closed triangles). Cells were then incubated for the indicated times in DMEM in the presence of the RNA synthesis inhibitor DRB 10 µg/ml. There was no significant difference in ABCA1 mRNA half-life between cholesterol-loaded and unloaded cells. Results represent mean +/- standard deviation from three different experiments.

ABCA1 gene transcription modulation by cholesterol and hydroxysterols: independent mechanisms?

In sterol depleted condition, SREBP can induce transcription of genes (LDL receptor) and repress it for others (MTP) [35]. Cholesterol may modulate the ABCA1 gene via the sterol response element binding protein (SREBP) pathway or through the formation of hydroxysterols that act as ligands for the LXR/RXR pathway. To examine whether cellular cholesterol can be converted to hydroxysterols to regulate ABCA1, we used geranylgeranyl-pyrophosphate (GGPP), an inhibitor of the interaction of the LXR/RXR complex with its target DR4 sequence [36]. It has been previously shown that GGPP, but not geranylgeraniol (GGOH) or other mevalonate metabolism pathway intermediates inhibits *in vitro* binding of the LXR/RXR complex to the DR4 sequences and the interaction of the dimer with their nuclear coactivator SRC-1 [37]. We first determined the optimum

dose of GGPP (5 µM) required to decrease ABCA1 transcription in HSF (not shown). It is noteworthy that GGPP is unable to inhibit ABCA1 transcription induced by 9CRA (10 µM) alone (data not shown), suggesting that GGPP blocks the binding of the LXR/RXR complex to its DR4 target sequence by blocking the LXR part of the heterodimer. GGPP 5 µM was then tested for its ability to inhibit ABCA1 transcription in cells treated with increasing concentrations of 22OH (0-5 µg/ml) or cholesterol (0-100 µg/ml) for 24 h. Figure 6 shows that higher doses of 22OH (Fig. 6A) and cholesterol (Fig. 6B) were required to activate ABCA1 transcription when cells were incubated in presence of 5 µM GGPP. These data show that ABCA1 transcription can be competitively inhibited by the addition of GGPP, suggesting that cholesterol requires conversion to hydroxycholesterols to induce ABCA1 transcription.

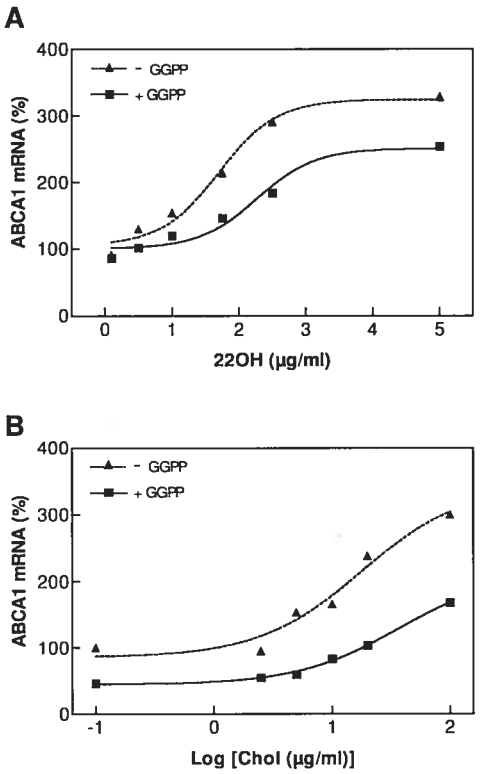

Figure 6. Geranylgeranyl pyrophosphate inhibition of 22(R)-hydroxycholesterol- and cholesterol-induced ABCA1 transcription. Competition effect of GGPP on the induction of ABCA1 transcription in HSF by (**A**) 22OH and (**B**) cholesterol. HSF were incubated for 24 h in DMEM/BSA 2 mg/ml in the presence (squares) or absence (triangles) (vehicle = methanol) of the LXR inhibitor, geranylgeranyl pyrophosphate (GGPP) 5 µM. The medium also contained increasing doses of (A) 22OH (0-5 µg/ml) or (B) cholesterol (0-100 µg/ml). ABCA1 mRNA is quantified and normalized for 28S loading. Results are expressed as a percentage of control conditions without GGPP and are representative of two different experiments. The x-axis in panel B) is logarithmic for increased clarity. Results are representative of two different experiments.

Discussion

We first found that regulation of ABCA1 transcription varies among tissues. Endothelial cells, for example, do not express high levels of ABCA1 nor efflux cholesterol to apoA-I [38]. To the opposite, HepG2 cells naturally secrete cellular cholesterol carriers such as apoA-I, apoB, apoE and bile acids, all possibly contributing to the high concentration of ^{3}H-cholesterol found in medium (Fig 2, efflux with BSA).

We also found that 8-Br-cAMP could not significantly increase ABCA1 expression in any cell type, in contrast to what was observed in J774 mouse macrophages. Even though another group has found that cAMP analogs can induce ABCA1 transcription in immortalized HSF [39], our results are similar to those obtained in other reports [18]. Interestingly, Cavelier et al. reported similar findings using a BAC containing the human ABCA1 transgene in mice. In their system, the human transgene was not regulated by cAMP analogs while the endogenous gene was [40]. In studies on ABCA1-mediated apoA-I binding [14] and cholesterol efflux [18], cAMP analogs are often used to induce expression of ABCA1 mRNA in mouse macrophages. Some authors propose the cAMP induction pathway as a potential target for therapeutic purposes [41]. However, our results raise the question of the relevance of cAMP as a physiological inducer of ABCA1 in humans. First, no study has ever demonstrated any correlation between endogenous cAMP increases and ABCA1 induction. Second, as our results show that cAMP does not induce ABCA1 expression, we conclude that cAMP is not a universal inducer of ABCA1 transcription. This does not exclude however post-translational regulation of ABCA1 by cAMP through protein phosphorylation [42].

Monocyte-derived macrophages and HSF show similar regulation patterns. However we decided to study the cholesterol homeostasis in fibroblasts, a well-known model to study Tangier disease. We found that cholesterol loading transcriptionally (Fig. 5) modulates the abundance of ABCA1 mRNA (Fig. 3A-C) and protein (Fig. 3D) in HSF. Using an ACAT inhibitor also found that cholesteryl esters do not modulate ABCA1 transcription. Cholesterol efflux to HDL_3 or apoA-I also decreased mRNA abundance, suggesting a close relationship between cellular sterol levels and transcription.

To determine the mechanism by which cholesterol induces ABCA1 transcription in HSF, we found that low doses of hydroxysterols modulate the ABCA1 gene in HSF and that an

inhibitor of LXR, GGPP, decreases ABCA1 basal and 22OH-induced transcription. This indicates that cholesterol conversion to a LXR ligand occurs in HSF and confirms an important role of the LXR/RXR pathway in transcription in HSF. The LXR nuclear factor is activated by hydroxysterol ligands, but not by cholesterol [43]; its endogenous ligand in HSF remains to be determined. A recent report suggests that 27-hydroxycholesterol is a good candidate ligand as cholesterol loading of skin fibroblasts from patients with cerebrotendinous xanthomatosis (cholesterol 27-hydroxylase deficiency) failed to increase ABCA1 transcription in HSF [44]. Thus it seems that hydroxysterols are acting as a proxy sensor for the presence of cholesterol. However, it remains to be determined whether or not cholesterol could modulate ABCA1 by mechanisms independent of conversion to hydroxysterols. Dual mechanisms of regulation by cholesterol and hydroxysterols are possible. One example of this is the promoter of the CETP gene that contains SRE elements [45] as well as LXR/RXR consensus sequences [46]. In our case, cellular cholesterol might modulate ABCA1 via three pathways: first, a direct effect on the ABCA1 promoter region, mediated via SRE-like sequences; second, through the inhibition of a transcriptional repressor, such as ZNF-202 [47], or third, through the inhibition of HMG CoA reductase and an increase in PPAR activity. It has been suggested that an increase in macrophage PPARα activity induces ABCA1 transcription [48] and that PPARγ agonists enhance LXR transcription which subsequently increase ABCA1 mRNA [49].

GGPP inhibits *in vitro* binding of the LXR/RXR complex to its target DR4 sequence [36] and was shown to decrease LXR/RXR-mediated induction of the CYP7A1 promoter activity in HepG2 cells [50]. However, GGPP is also a substrate for the prenylation of proteins, especially small G-proteins [51]. In their study, Gan et al. used an inhibitor of geranylgeranyl transferase, the enzyme transfering GGPP onto Rho proteins, causing a subsequent increase in ABCA1 transcription. They conclude that the prenylation of small G-proteins with GGPP is also involved in the regulation of ABCA1 transcription [37]. However, at the dose selected here, the effect of GGPP is inhibitory and therefore likely to be due to direct inhibition of LXR.

ABCA1 is differentially regulated in human cell types. Even though endothelial cells constitute an important tissue for atherosclerosis, low level of ABCA1 and absence of cholesterol efflux to delipidated apoA-I suggests a minimal (if no) contribution to formation of pre-beta particles [38]. Also, macrophages contribute only mildly to HDL levels [24].

Our study revealed that sterols modulate ABCA1 transcription in human skin fibroblasts. We hypothesize that conversion of cholesterol to hydroxycholesterol contributes to this regulation, but cannot exclude an independent action of free cholesterol. Fibroblasts constitute an important tissue of the body and might contribute importantly to the early steps of HDL formation. However, the importance of the liver in the generation of poorly lipidated apoA-I particles is well established in virtue of its ability to synthesize apoA-I and ABCA1. Studies on cholesterol efflux in Tangier fibroblasts have shown that absence of functional ABCA1 causes low efflux, and consequently a hypercatabolism of immature particles [52]. We have previously shown that plasma HDL-C levels correlate with cellular cholesterol efflux and that PKA can modulate the ABCA1-dependent efflux at the post-transcriptional level [42]. Taken together, these findings open the possibility of pharmacological tissue-specific modulation of the ABCA1 efflux pathway for therapeutic purposes.

Acknowldgements

A doctoral research award from the Heart and Stroke Foundation of Canada supports M. Denis. The work is supported by CIHR grant MOD 15042 and CHIR-Rx&D grant DOP 48045. J. Genest holds a CIHR-Novartis chair at McGill University.

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Chapitre IV

Article #2 *

1. Contexte de travail

Il était entendu que l'apoA-I génère un efflux de cholestérol et de phospholipides et que cet efflux est dépendant de la présence d'ABCA1. Toutefois, la nature de l'interaction entre apoA-I et ABCA1 restait indéterminée. Deux modèles s'opposaient: l'un était basé sur une interaction indirecte, l'autre sur un contact protéine/protéine. Aussi, les modèles proposés dans la littérature supposaient que les particules résultantes de cette interaction auraient une migration pré-β, avec une structure discoïdale.

Afin de comprendre le mécanisme de formation des HDL, il fut primordial de déterminer la nature de l'interaction de l'apoA-I avec l'ABCA1, ainsi que de caractériser le produit de cette interaction.

C'est dans ***le but d'étudier l'interaction entre l'apoA-I et l'ABCA1 et de caractériser les particules qui sont générées*** que le travail a été entrepris.

Dans l'article suivant,

Maxime Denis a fait la majeure partie du travail et a co-écrit le manuscrit
Bassam Haidar a fait la figure 1b
Michel Marcil a dessiné la figure 7
Michel Bouvier a participé à la supervision
Larbi Krimbou a co-écrit le manuscrit et a aidé à faire les gels (fig 5 et 6)
Jacques Genest a supervisé le travail

*** Note de l'auteur :** Cet article mis en page sous forme "pdf" est disponible en annexe

2. Article:

"Molecular and Cellular Physiology of Apolipoprotein A-I Lipidation by the ATP-binding Cassette Transporter A1 (ABCA1)"

(Published in *The Journal of Biological Chemistry* (2004) Vol 279 No 9, pp 7384-7394)

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Running title: Lipidation of apoA-I by ABCA1

ABSTRACT

The dynamics of ABCA1-mediated apoA-I lipidation were investigated in intact human fibroblasts induced with 22 (R)-hydroxycholesterol and 9-*cis*-retinoic acid (stimulated cells). Specific binding parameters of ^{125}I-apoA-I to ABCA1 at 37°C were determined: K_d = 0.65 µg/mL, B_{max} = 0.10 ng/µg cell protein. Lipid-free apoA-I inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than preβ_1-LpA-I, reconstituted HDL particles r(LpA-I), or HDL_3 (IC_{50} = 0.35 ± 1.14, apoA-I; 1.69 ± 1.07, preβ_1-LpA-I; 17.91 ± 1.39, r(LpA-I); and 48.15 ± 1.72 µg/mL, HDL_3). Treatment of intact cells with either phosphatidylcholine-specific phospholipase C or sphingomyelinase affected neither ^{125}I-apoA-I binding nor ^{125}I-apoA-I/ABCA1 cross-linking. We next investigated the dynamics of apoA-I lipidation by monitoring the kinetic of apoA-I dissociation from ABCA1. The dissociation of ^{125}I-apoA-I from normal cells at 37°C was rapid ($t_{½}$ = 1.4 ± 0.66 h; n = 3) but almost completely inhibited at either 15°C or 4°C. A time-course analysis of apoA-I-containing particles released during the dissociation period showed nascent apoA-I-phospholipid complexes that exhibited α-electrophoretic mobility with a particle size ranging from 9 to 20 nm (designated α-LpA-I-like particles), whereas lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells was unable to form such particles. These results demonstrate that: 1) the physical interaction of apoA-I with ABCA1 does not depend on membrane phosphatidylcholine or sphingomyelin; 2) the association of apoA-I with lipids reduces its ability to interact with ABCA1; and 3) the lipid translocase activity of ABCA1 generates α-LpA-I-like particles. This process plays *in vivo* a key role in HDL biogenesis.

Keywords: ApoA-I lipidation, ABCA1, lipid efflux, HDL

ABBREVIATIONS

2D-PAGGE, two-dimensional polyacrylamide non-denaturing gradient gel electrophoresis; ABCA1, ATP binding cassette AI; apo, apolipoprotein; BSA, Bovine serum albumin; CETP, cholesteryl ester transfert protein; FHD, Familial HDL deficiency; HDL, high density lipoprotein; H-TGL, hepatic lipase; LCAT, lecithin : cholesterol acyl transferase; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospolipase C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; r(LpA-I), reconstituted HDL particles; RCT, reverse cholesterol transport; SM-ase, sphingomyelinase; SM, sphingomyelin; SR-BI, scavenger receptor class B type I; TD, Tangier disease. TLC, thin layer chromatography.

INTRODUCTION

Apolipoprotein (apo) A-I binding to the extracellular domain of ABCA1 results in the activation of apoA-I lipidation, a key step in reverse cholesterol transport (RCT) process, one of the several proposed mechanisms by which HDL may protect against atherosclerotic vascular disease (1-3).

The molecular interaction of apoA-I with ABCA1 promotes cholesterol efflux from peripheral cells and macrophages and is critical for the initial formation of HDL-particles (1). The importance of ABCA1 in the lipidation of apoA-I has been strikingly demonstrated by the identification of mutations at the ABCA1 gene locus as the molecular defect of Tangier Disease (TD) and Familial HDL Deficiency (FHD) (4,5). These patients are characterized by extremely low HDL-cholesterol levels, caused by inadequate transport of cellular cholesterol and phospholipids to the extracellular space, leading to hypercatabolism of lipid-poor nascent HDL particles (6).

ApoA-I has been shown to interact with many proteins including high-density lipoprotein binding protein (HBP, vigilin), HB2 (7), annexin I, annexin VII (8), fibronectin, collagen I (9,10), and the human β-chain of ATP synthase (11). However, the physiological significance of these interactions remains unknown. On the other hand, it is well established that apoA-I binds to the scavenger receptor class B type I (SR-BI) (12) which participates in selective uptake of HDL-derived cholesteryl esters, but so far no role for SR-BI in apoA-I-mediated lipid efflux has been found.

Although several studies have suggested a molecular interaction between apoA-I and ABCA1 at the cell surface (13-15), the role of ABCA1 as a candidate apoA-I receptor is still a matter of debate. At least two different mechanisms are proposed for this interaction. First, it is reported that a direct protein-protein interaction occurs between apoA-I and ABCA1 on the basis of chemical cross-linking experiments (13). A second hypothesis has been proposed suggesting an interaction between apoA-I and lipid domains in the cell membranes formed by the phospholipid translocase activity of ABCA1 (14). Indeed, studies by Remaley *et al.* (16,17) have shown that a majority of the plasma apolipoproteins containing lipophilic class A amphipathic helices can also promote lipid efflux and bind to ABCA1. Furthermore, the amphipathic helix was found to be a key structural motif for peptide-mediated lipid efflux from ABCA1.

Without knowledge of specific binding parameters of apoA-I-containing particles to ABCA1, it is not possible to predict whether ABCA1 might function as a significant receptor for apoA-I in the presence of other apolipoproteins, which demonstrate affinity for

the same protein. A recent study by Basso *et al.* (18) demonstrating that the hepatic expression of ABCA1 is an important source of plasma HDL-C has stimulated our interest for apoA-I lipidation in peripheral cells. In the present study, experiments were directed at defining the mechanism by which apoA-I is lipidated by ABCA1 and how the formation of the apoA-I/ABCA1 complex can be affected by apoA-I conformation within discoidal and spherical HDL particles, by specific hydrolysis of plasma membranes phospholipids, or by naturally occurring mutants of ABCA1. In addition, the dynamics of apoA-I lipidation were investigated by determining the kinetic parameters of apoA-I/ABCA1 dissociation and the characterization of apoA-I-containing particles generated during this process.

EXPERIMENTAL PROCEDURES

Patients selection — For the present study, we selected fibroblasts from 3 normal control subjects and 1 patient with TD (homozygous for Q597R at the ABCA1 gene). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Centre. Separate consent forms for blood sampling, DNA isolation and skin biopsy were provided.

Cell culture — Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearm of patients and healthy control subjects and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum (FBS).

Human plasma apoA-I — Purified plasma apoA-I (Biodesign) was resolubilized in 4M guanidine HCL and dialyzed extensively against Tris buffer, (10 mM Tris, 150 mM NaCl; pH 8.2). Freshly resolubilized apoA-I was used within 48 h.

ApoA-I Binding Assay — ApoA-I was iodinated with ^{125}Iodine by IODO-GEN® (Pierce) to a specific activity of 800-2500 cpm/ng apoA-I. Cells were grown on 24-well plates and were stimulated or not with 2.5 µg/mL 22-(R) hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h. Cells were then incubated at 37°C with ^{125}I-apoA-I in DMEM/BSA (1 mg/mL) as specified for each experiment in the presence or absence of a 30-fold excess of unlabeled apoA-I, to subtract the non-specific binding. The cells were then washed rapidly two times with ice cold PBS/BSA, two times with cold PBS and lysed with 0.1 N NaOH. The amount of bound iodinated ligand was determined by gamma counting.

Chemical cross-linking and immunoprecipitation (IP) analysis — Chemical cross-linking was performed as described by Wang *et al.* (19) with a minor modification. Fibroblasts were grown to confluence in 100 mm diameter dishes and then stimulated or not with 2.5 µg/mL 22-(R) hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h in DMEM / BSA. Cells were incubated in the presence or absence of either 3 µg/mL of unlabeled apoA-I or 10 µg/mL of ^{125}I-apoA-I in DMEM / BSA for 1 h at 37°C. Cells were then placed on ice for 15 min and washed three times with PBS. DSP (cross-linker agent) was dissolved immediately before use in dimethyl sulfoxide (DMSO) and diluted to 500 µM with PBS. Eight mL of DSP solution was added in each well. Cells were then incubated at room temperature for 1 h; the medium was removed, and the cells were washed twice with PBS. Cells were lysed at 4°C with IP buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton-X 100 (Invitrogen) and the suspension was allowed to stand for 30 min at 4°C in presence of a protease inhibitor cocktail (Roche Diagnostics).

ApoA-I / ABCA1 complex was immunoprecipitated with an affinity purified polyclonal anti-ABCA1 antibody (Novus Biologicals) as previously described (20). After SDS-gel electrophoresis either apoA-I or ABCA1 were detected by immunopurified polyclonal anti-apoA-I antibody (Biodesign) or affinity-purified human anti-ABCA1 antibody (Novus). The presence of labeled ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography using XAR-2 Kodak film.

Quantitative cross-linking of apoA-I to ABCA1 — Fibroblasts were grown to confluence in 100 mm diameter dishes and then stimulated for 20 h. Cells were incubated at 37°C for 1 h in the presence or absence of 5 U/mL PC-PLC or 0.4 U/mL SM-ase. After washing to remove phospholipases, cells were incubated with 10 µg/mL of ^{125}I-apoA-I (1500 to 2500 cpm/ng) in the presence or absence of a 20-fold excess of unlabeled apoA-I. Cells were then placed on ice for 15 min and washed three times with PBS, and then cross-linking with DSP was performed as described above. Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 µg total protein) were incubated with 10 µL of affinity-purified human anti-ABCA1 antibody for 20 h at 4°C, followed by the addition of Protein A bound to sepharose (30 µL) as we have described previously (21). The amount of bound iodinated apoA-I to ABCA1 in the immunoprecipitates was determined by gamma counting. ABCA1 mutant (Q597R) was used as a negative control.

Dissociation of specifically bound ^{125}I-apoA-I from intact cells — Fibroblasts were grown to near confluence in 24-well plates and then stimulated with 2.5 µg/mL 22-(R) hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h in DMEM/BSA. The cells were incubated for 2 h at 37°C with 10 µg/mL of ^{125}I-apoA-I in the presence of 1 mg/mL BSA. For non-specific binding determination, cells were incubated with a 30-fold excess of unlabeled apoA-I. After washing to remove unbound ^{125}I-apoA-I, 0.5 mL of DMEM was added, and the plates were immediately incubated at 37°C, 15°C, or 4°C for the indicated times. The medium was then collected, cells were lysed in 0.1 N NaOH, and the radioactivity in the medium and in the cells was determined by gamma counting.

Cellular lipid efflux and lipid labeling — Phospholipid and cholesterol efflux were determined as previously described (3) with minor modifications. Briefly, 50 000 cells were seeded in 12-well plates. At mid-confluence, the cells were labeled with 0.2-5 µCi/mL ^{3}H-choline (Perkin Elmer) or 0.2-5 µCi/mL ^{3}H-cholesterol (Perkin Elmer) for 48 h. At confluence, cells were cholesterol-loaded (20 µg/mL) for 24 h. During a 24 h equilibration period, cells were stimulated or not with 2.5 µg/mL of 22 (R)-hydroxycholesterol and 10 µM of 9-*cis*-retinoic acid for 20 h. Phospholipid or cholesterol efflux were determined at

either 2 h or 24 h with 10 µg/mL apoA-I. Cellular lipid efflux was determined as follow: ^{3}H cpm in medium/(^{3}H cpm in medium + ^{3}H cpm in cells); the results were expressed as % of total radiolabeled phospholipids or cholesterol. Cell phospholipids were also labeled with ^{32}P-orthophosphate as follows: fibroblasts from control subject were grown to confluence in 100 mm or 150 cm diameter dishes and were incubated for 72 h with 300-1500 µCi of ^{32}P-orthophosphate mixed with DMEM. The cells were stimulated as described above before incubation with lipid-free apoA-I as specified for each experiment.

Separation of lipoproteins by two-dimensional non-denaturing gradient gel electrophoresis (2D-PAGGE) — ApoA-I-containing particles were separated by 2D-PAGGE, as previously described (22,23). Briefly, samples (30-100 µL) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4°C) and in the second dimension (according to the size) by 5-23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4 °C). Iodinated high molecular weight protein mixture (7.1 nm to 17.0 nm, Pharmacia) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes (Hybond ECL, Amersham). ApoA-I-containing particles were detected by incubating the membranes with immunopurified polyclonal anti-apoA-I antibody (Biodesign) labeled with ^{125}I. The presence of labeled ^{125}I-apoA-I or ^{32}P-phospholipids were directly detected by autoradiography using XAR-2 Kodak film.

Preparation of reconstituted HDL particles (rLpA-I) — Complexes comprising apoA-I, POPC, and cholesterol were prepared using the sodium cholate dialysis method (24). ApoA-I/POPC/cholesterol molar ratio of 1:100:5 was used in this experiment. r(LpA-I) particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 50,000, Amicon) to discard any lipid-free apoA-I or proteolytic peptides. ApoA-I-lipid complex formation was verified by analysis with 2D-PAGGE.

Preβ$_1$-LpA-I purification from plasma — Preβ$_1$-LpA-I was purified from freshly drawn venous blood under nondenaturing conditions as described by Kunitake *et al.* (25) with the following modifications. Typically, blood is drawn into a tube containing 1mM sodium EDTA, 0.02% NaN_3, 2 mM DTNB and cooled immediately on ice. Plasma is separated by low speed centrifugation (1,800 *x g*, 30 min) and aliquots (20 mL) were subjected to human immunopurified anti-apoA-I antibody (12171-21A, Genzyme Corp) -coupled sepharose column (23,26). ApoA-I-containing fractions were then dialyzed and concentrated. Samples were separated by agarose gel electrophoresis, and the preβ-migrating region was excised out. Agarose gel pieces containing the pre-β-migrating

region were placed at the top of 3-26% non-denaturing gradient gels, as previously described (27). An immunoblot of apoA-I-containing lipoproteins separated by 2D-PAGGE gels was used as a template to localize preβ_1-LpA-I particles which are recovered from the gels by electroelution. Preβ_1-LpA-I particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 50,000, Amicon) to discard any lipid-free apoA-I or proteolytic peptides. The integrity of isolated plasma preβ_1-LpA-I fraction was verified by 2D-PAGGE.

RESULTS

In the present study, we have examined the binding of ^{125}I-apoA-I to ABCA1 in normal cultured human fibroblasts. To determine the specific binding of ^{125}I-apoA-I to ABCA1, binding studies were performed in fibroblasts in which ABCA1 was induced with 22 (R)-hydroxycholesterol and 9-*cis*-retinoic acid (stimulated cells), as well as in unstimulated cells. As shown in Fig. 1A, a marked and consistent increased binding of ^{125}I-apoA-I to stimulated cells was measured. However, significant binding was also observed in unstimulated cells. This is presumably due to basal level of ABCA1 expression and the presence of other apoA-I binding sites at the cell surface. We have not been able to detect any SR-BI receptor presence in fibroblasts as compared to hepatocytes as examined by gel electrophoresis of cellular membranes fraction followed by immunoblotting with an anti-SR-BI antibody (data not shown). The specific binding curve and the binding parameters K_d and B_{max} for apoA-I/ ABCA1 interactions were determined by subtracting the binding values for the unstimulated cells from the corresponding values from stimulated cells. In the present binding assay apoA-I binds to ABCA1 (ABCA1 specific) with relatively high affinity (K_d = 0.65 ± 0.20 µg/mL), and the binding was saturable (B_{max}= 0.10 ± 0.05 ng/µg cell protein) (Fig. 1A). Maximum specific binding of apoA-I to ABCA1 was reached in less than 30 min and remained constant for the remaining of 2 h of the experiment (data not shown). To ensure that the binding parameters obtained reflect specific increased of ^{125}I-apoA-I/ABCA1 association in stimulated cells, the cross-linking of apoA-I to ABCA1 was examined. As shown in Fig. 1B, apoA-I forms a complex with ABCA1. Furthermore, stimulation of cells lead to an increase of both cellular ABCA1 expression and apoA-I/ABCA1 cross-linking compared to unstimulated cells. At the same time, phospholipid and cholesterol efflux were increased in stimulated cells, as shown in Fig. 1C. In order to verify that the specific association of ^{125}I-apoA-I with ABCA1 was dependent on the temperature, stimulated cells were incubated with 10 µg/mL of ^{125}I-apoA-I for 2 h at either 37°C, 20°C or 4°C, and then specific ^{125}I-apoA-I cell association was determined as described above. ^{125}I-apoA-I association with stimulated cells showed remarkable temperature dependence (100 ± 2%, 29 ± 4% and 13 ± 2%; 37°C, 20°C and 4°C, respectively). Results are expressed as percent of the incubation at 37°C (100%).

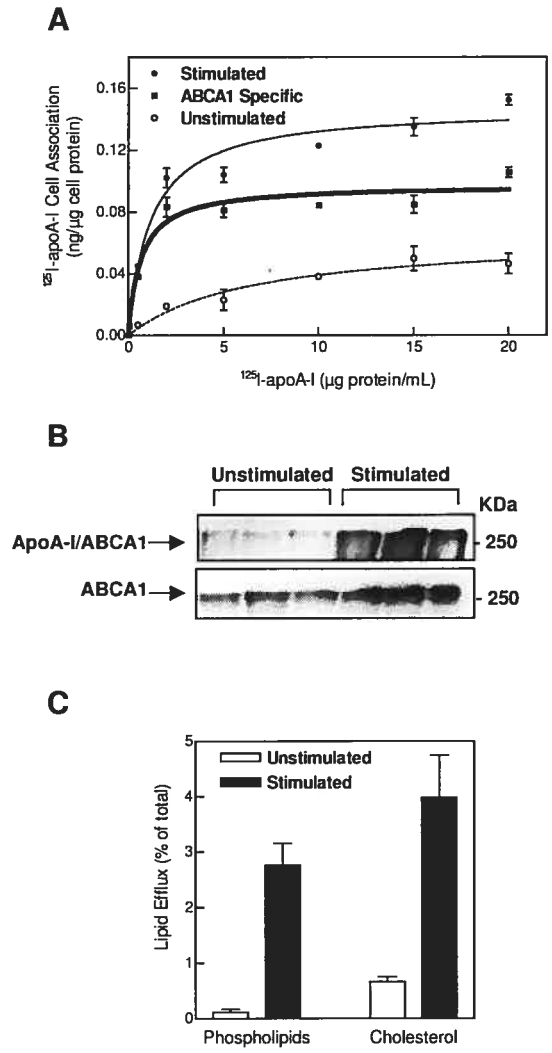
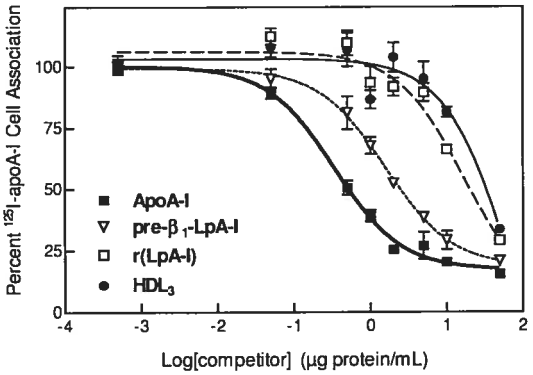


Figure 1. Effect of 22 (R)-hydroxycholesterol and 9-*cis*-retinoic acid on ^{125}I-apoA-I cell association, apoA-I/ABCA1 complex formation and cellular lipid efflux. ***A***, normal control fibroblasts were plated in 24-well plates and stimulated or not with 2.5 µg/mL 22 (R)-hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h. Cells were then incubated for 2 h at 37°C with increasing amounts of ^{125}I-apoA-I (0, 2.5, 5, 10, 15, 20 µg/mL). Non-specific binding was determined for both stimulated and unstimulated cells in the presence of a 30-fold excess of unlabeled apoA-I. The specific binding curve (ABCA1 specific) was determined by subtracting the binding values for the unstimulated cells from the corresponding values for stimulated cells. Binding parameters of ^{125}I-apoA-I to ABCA1 were analyzed using Graph Pad Prism 4.00 software. ***B***, stimulated and unstimulated fibroblasts were incubated with 3 µg/mL apoA-I at 37°C for 1 h. Cells were washed 2 times with cold PBS and exposed to the DSP cross-linker for 1 h at room temperature. ApoA-I/ABCA1 complexes were immunoprecipitated with an anti-ABCA1 antibody and run on 6% SDS-PAGE. ApoA-I associated with ABCA1 (*upper panel*) or ABCA1 itself (*lower panel*) were detected by immunoblotting with an anti-apoA-I antibody or an anti-ABCA1 antibody. ***C***, stimulated and unstimulated normal cells were radiolabeled with either ^{3}H-cholesterol or ^{3}H-choline and incubated with 10 µg/mL of apoA-I at 37°C for 2 h. Phospholipid and cholesterol efflux were determined as described in "Experimental Procedures". Bars represent mean ± SD of an experiment performed in triplicate.

It is well established that the conformation of apoA-I within HDL particles is affected by its association with lipid molecules. It was therefore of interest to determine whether apoA-I conformation/organization within particles would affect its interaction with ABCA1. Competition assays were performed to determine the ability of preβ$_1$-LpA-I, as well as discoidal reconstituted HDL r(LpA-I) and spherical HDL particles (HDL$_3$), to compete for the binding of ^{125}I-apoA-I to ABCA1 in stimulated cells. As shown in Fig. 2A, lipid-free apoA-I inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than either isolated plasma preβ$_1$-LpA-I, reconstituted HDL particles r(LpA-I) (11-12 nm of diameter), or native HDL$_3$ (IC_{50} = 0.35 ± 1.14, apoA-I; 1.69 ± 1.07, preβ$_1$-LpA-I; 17.91 ± 1.39, r(LpA-I); and 48.15 ± 1.72 µg/mL, HDL$_3$). Control experiments were conducted to examine whether the apparent decrease in cell binding of the labeled apoA-I may be due to the ^{125}I-apoA-I binding to different competitor particles instead of the cells. An experiment was therefore carried out in which either r(LpA-I) or HDL$_3$ particles were incubated with ^{125}I-apoA-I under simillar conditions used for apoA-I binding assay and then the samples were separated by fast protein liquid chromatography (FPLC). No significant amount of ^{125}I-apoA-I was found associated with r(LpA-I) or HDL$_3$ (data not shown), supporting our results shown in Fig. 2A. To verify the integrity of competitors particles, either isolated preβ$_1$-LpA-I, lipid-free apoA-I, r(LpA-I) or plasma were separated by 2D-PAGGE and apoA-I was detected with immunopurified polyclonal anti-apoA-I antibody labeled with ^{125}I, as shown in Fig. 2B.

A



Competitor	IC_{50} (µg protein/mL)
ApoA-I	0.35 ± 1.14
pre- $_1$-LpA-I	1.69 ± 1.07
r(LpA-I)	17.91 ± 1.39
HDL_3	48.15 ± 1.72

B

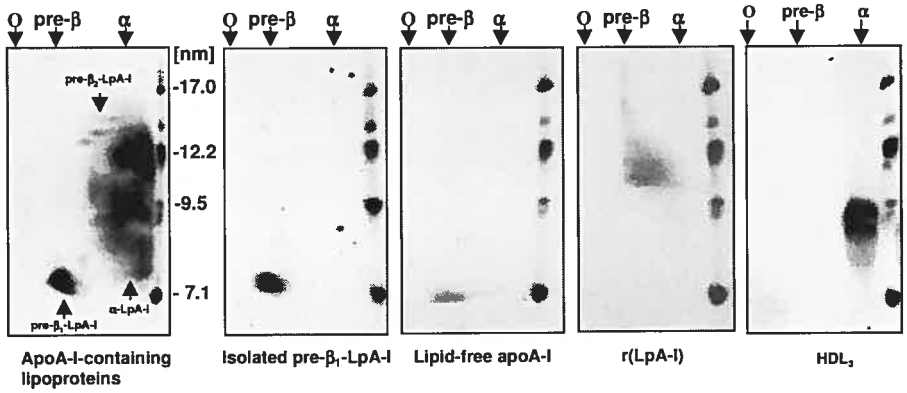


Figure 2. Ability of preβ$_1$-LpA-I particles, reconstituted HDL particles r(LpA-I) and native HDL_3 to interact with ABCA1. ***A,*** normal cells were plated in 24-well plates and stimulated for 20 h. Cells were then incubated with 2 µg/mL of ^{125}I-apoA-I for 2 h at 37°C with increasing amounts of either plasma isolated preβ$_1$-LpA-I, reconstituted HDL r(LpA-I), native HDL_3 and unlabeled apoA-I (0, 0.05, 0.5, 1, 2, 5,10, 50 µg protein/mL). Cells were then washed rapidly three times with ice cold PBS/BSA and then PBS alone. ^{125}I-apoA-I associated with cells was determined as described in "Experimental Procedures". The values shown represent the mean ± SD from triplicate wells. The 100% of control value measured in the absence of competitors was 0.8 ng of apoA-I/µg cell protein. Similar results were obtained in four independent experiments. Values of IC_{50} shown were determined using the Graph Pad Prism 4.00 software. ***B,*** either plasma isolated preβ$_1$-LpA-I, reconstituted HDL r(LpA-I), native HDL_3 or plasma were separated by 2D-PAGGE and apoA-I was detected with immunopurified polyclonal anti-apoA-I antibody labeled with ^{125}I. Molecular size markers are indicated on the right side of each gel.

Because the lipid binding characteristics of apoA-I have been proposed to be important in the apoA-I/ABCA1 interaction (14,15,28), the question was raised whether the binding of apoA-I to ABCA1 was depended on the presence of lipids at the cell surface. Stimulated cells were incubated for 60 min at 37°C in the presence or absence of either 5 U/mL phosphatidylcholine-specific phospholipase C (PC-PLC) or 0.4 U/mL sphingomyelinase (SM-ase). To assess how effectively phospholipids were removed by phospholipases treatment, the cells were labeled with [^{3}H]–choline and the lipids separated by TLC and counted. PC-PLC and SM-ase treatment digested greater than 65% of phosphatidyl [^{3}H]-choline and 80% of [^{3}H]-sphingomyelin, respectively. Cells were then incubated with 10 µg/mL of ^{125}I-apoA-I for 2 h at 37°C and specific ^{125}I-apoA-I binding was determined as described above. As shown in Fig. 3A, no significant effect of phospholipases treatment on the ^{125}I-apoA-I binding level was observed. In order to further verify that the interaction of apoA-I with ABCA1 was not dependent on the presence of plasma membranes phosphatidylcholine or sphingomyelin, we determined whether the cross-linking of apoA-I to ABCA1 could be affected by phospholipases treatment. Quantitative cross-linking of apoA-I to ABCA1 was performed as described in "Experimental Procedures". As shown in Fig. 3B (lower panel), treatment of intact cells with phospholipases did not affect significantly ^{125}I-apoA-I cross-linking to ABCA1. The presence of a 20-fold excess of unlabeled apoA-I (200 µg/mL) reduced the cross-linking of ^{125}I-apoA-I to ABCA1 by 78% of control. In addition, ABCA1 mutant (Q597R) that has been shown previously to not cross-link to apoA-I (13) was used as a negative control for the present experiment and showed no binding or cross-linking to ABCA1 (Fig. 3A,B). To ensure that the immunoprecipitates contained only ^{125}I-apoA-I/ABCA1 complex, immunoprecipitated samples were analysed by 4-22.5% SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3B (upper panel), only ^{125}I-apoA-I/ABCA1 complex was detected in immunoprecipitated samples. Also, to rule out the possibility that treatment with phospholipases might induce membrane aggregation or affect ABCA1 protein structure, which may trap apoA-I and result in non-specific cross-linking, we examined the effect of phospholipases treatment on the cross-linking of ^{125}I-apoA-I to ABCA1 mutant (Q597R). As shown in Fig. 3B (upper panel), ^{125}I-apoA-I did not cross-link to Q597R mutant whether treated with PC-PLC, SM-ase or left intact.

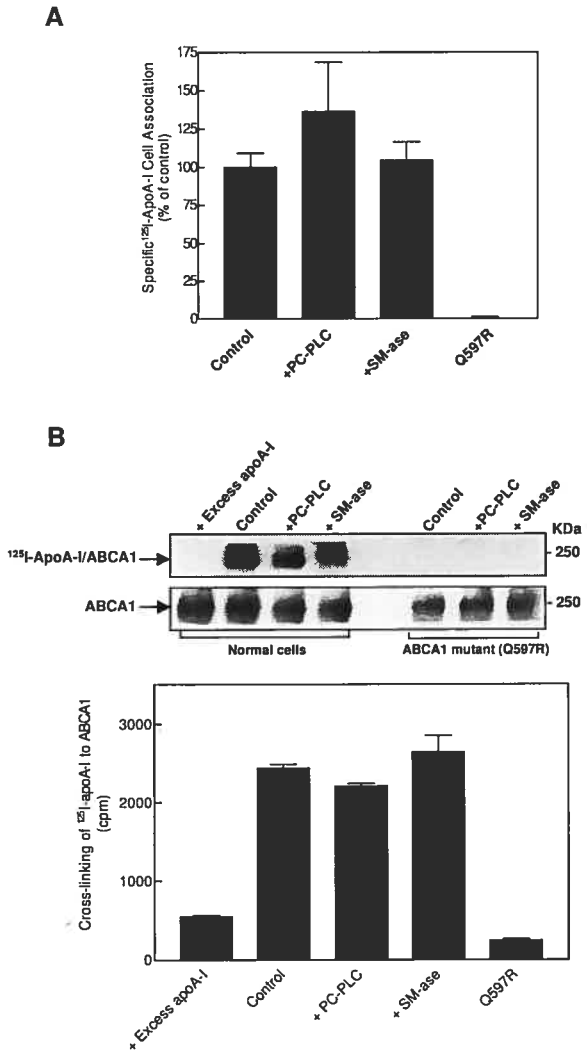


Figure 3. Effect of phospholipases treatment on apoA-I/ABCA1 interactions. ***A,*** stimulated cells were incubated for 60 min at 37°C in the presence or absence of 5 U/mL PC-PLC or 0.4 U/mL SM-ase. Cells were then incubated with 10 µg/mL of ^{125}I-apoA-I for 2 h at 37°C. Specific ^{125}I-apoA-I binding was determined as described in Fig. 1A. Control value (100%) represent 24.01 ng of apoA-I/mg cell protein. ABCA1 mutant (Q597R) was used as a negative control. ***B,*** **upper panel**, intact stimulated normal or Q597R cells in 100 mm diameter dishes were incubated or not with PC-PLC or SM-ase as described above and then incubated with 10 µg/mL of ^{125}I-apoA-I for 1 h at 37°C in the presence or absence of a 20-fold excess of unlabeled apoA-I (200 µg/mL) . Cross-linking with DSP was performed as described above. Samples containing- ^{125}I-apoA-I cross-linked to ABCA1 (200 µg total protein) were incubated with 10 µL of affinity-purified human anti-ABCA1 antibody for 20 h at 4°C, followed by addition of Protein A bound to separose (30 µL). Immunoprecipitated samples were separted on 4-22.5% SDS-polyacrylamide gel electrophoresis and ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography. The ABCA1 protein was detected on the same membrane by an anti-ABCA1 antibody. **Lower panel**, intact normal cells were incubated or not with PC-PLC or SM-ase as described above and then incubated with 10 µg/mL of ^{125}I-apoA-I for 1 h at 37°C in the presence or absence of a 20-fold excess of unlabeled apoA-I. Quantitative cross-linking of ^{125}I-apoA-I to ABCA1 was performed as described in "Experimental Procedures". The amount of bound iodinated apoA-I to ABCA1 in the immunoprecipitates was determined by gamma counting. Results shown are representative of two different independent experiments.

We initially hypothesized that any specific apoA-I dissociation from ABCA1 would be associated with a significant increase in apoA-I lipidation state. To better understand the mechanism by which apoA-I was lipidated by ABCA1, the kinetics of the dissociation of apoA-I from ABCA1 were investigated in stimulated cells. Fig. 4A depicts the time-course of the dissociation of bound ^{125}I-apoA-I from stimulated normal cells at 37°C. The dissociation of ^{125}I-apoA-I from ABCA1 at 37°C was rapid ($t_{½}$ =1.4 ± 0.66 h; n = 3). In contrast, ^{125}I-apoA-I dissociation from ABCA1 was almost completely inhibited at either 4°C or 15°C (Fig. 4A). Practically all radioactivity that disappeared from the cell surface appeared as intact ^{125}I-apoA-I in the medium (more than 95% of the radioactivity released to the medium was precipitated by 10% trichloroacetic acid).

To further investigate the relationship between apoA-I dissociation from ABCA1 and apoA-I-mediated cellular cholesterol efflux in our stimulated cell culture system, the kinetics of cholesterol efflux in stimulated cells was determined in the presence of 10 µg/mL of apoA-I (saturating binding concentrations). As shown in Fig. 4B, apoA-I-mediated cholesterol efflux reached saturation after a 16 h incubation period. In addition, to ensure that cholesterol efflux was ABCA1-dependent in our cell culture system, apoA-I-mediated cholesterol efflux from ABCA1 mutant (Q597R) cells was also monitored.

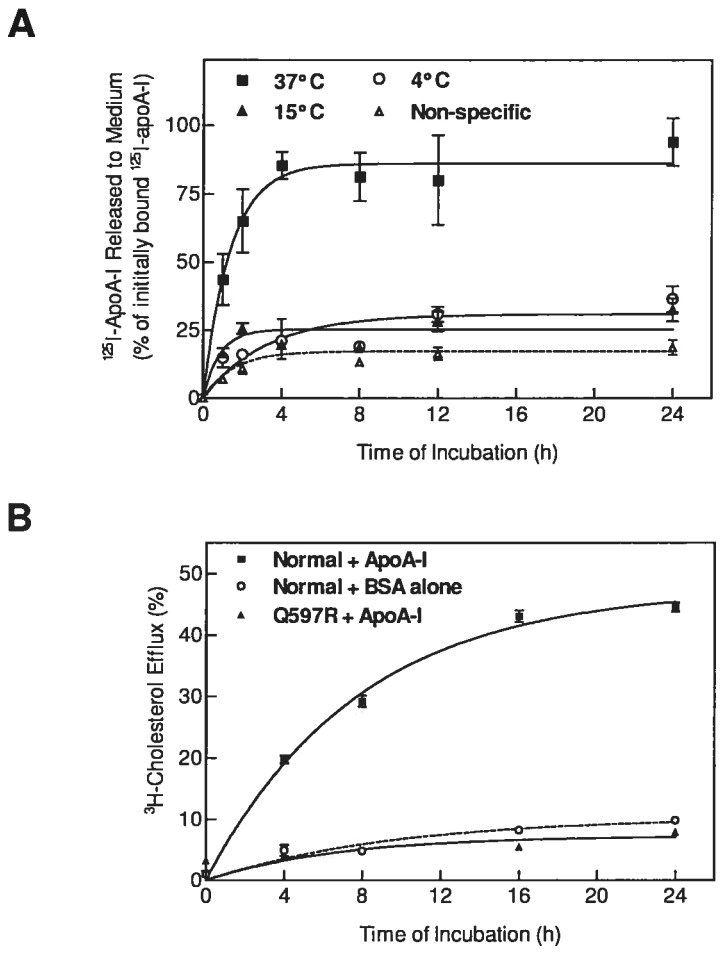


Figure 4. Dissociation of ^{125}I-apoA-I from stimulated fibroblasts and the kinetics of ABCA1-dependent cholesterol efflux. ***A,*** stimulated cells in 24-well plates were incubated with 10 µg/mL of ^{125}I-apoA-I for 2 h at 37°C. Non-specific binding was determined in the presence of a 30-fold excess of unlabeled apoA-I and shown as the non-specific. After washing to remove unbound ^{125}I-apoA-I, 0.5 mL of DMEM was added and the plates were immediately incubated at either 37°C, 15°C or 4°C. At various time points, the radioactivity appearing in the medium was determined. Values represent the mean ± SD from triplicate wells. The initial binding value measured in t = 0 h was 0.24 ± 0.08 ng of apoA-I/µg cell protein. Similar results were obtained from 2 other control fibroblast cell lines. ***B,*** stimulated normal or Q597R cells were radiolabeled with ^{3}H-cholesterol and incubated with 10 µg/mL of apoA-I or 1 mg/ mL of BSA at 37°C for the indicated time points. Cholesterol efflux was determined as described in "Experimental Procedures". Values represent the mean ± SD from triplicate wells. Results shown are representative of four different independent experiments

In order to investigate the nature of apoA-I-containing particles generated by ABCA1 activity, stimulated cells from either normal or from TD (Q597R) subjects in 100 mm diameter dishes were incubated with 10 µg/mL of ^{125}I-apoA-I in 8 mL of DMEM for 24 h at 37°C. The medium was concentrated and ^{125}I-apoA-I-containing particles were separated by 2D-PAGGE. As shown in Fig. 5 (panel B), apoA-I-containing particles generated by stimulated normal cells exhibited α-electrophoretic mobility with a particle diameter ranging from 9 to 20 nm, however, a significant amount of apoA-I was detected in the pre-β-region. In contrast, lipid-free apoA-I incubated with stimulated mutant Q597R cells was unable to form such particles (panel C), which had a molecular diameter and charge similar to the lipid-free apoA-I incubated in the same conditions without cells (panel A).

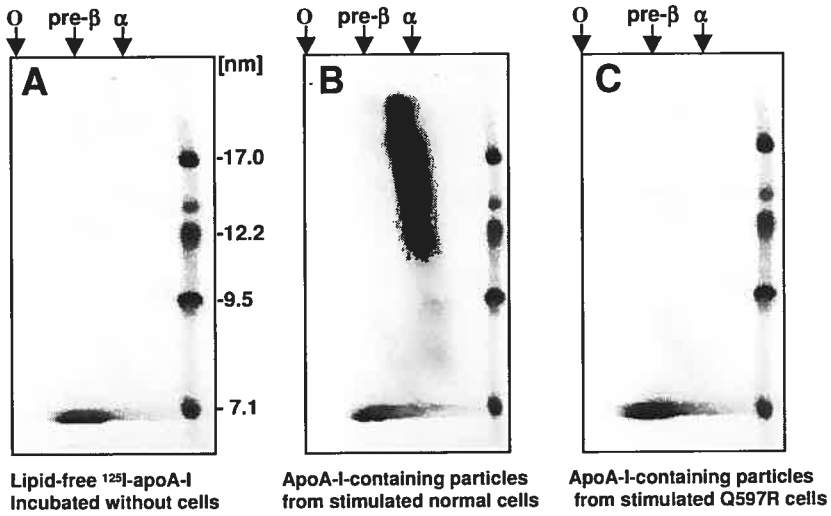


Figure 5. Analysis of lipid-free apoA-I charge and molecular diameter after incubation with either stimulated normal or ABCA1 mutant cells. ^{125}I-apoA-I was incubated in DMEM / BSA for 24 h at 37°C without cells (***A***) or with both stimulated normal and Q597R cells (***B and C***, respectively) for 24 h at 37°C. Samples were separated by 2D-PAGGE and ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film. Molecular size markers are indicated on the right side of each gel.

To further characterize apoA-I-containing particles released specifically from ABCA1 during the dissociation period, stimulated cells in 150 mm diameter dishes were incubated with 10 µg/mL ^{125}I-apoA-I for 2 h at 37°C. After washing to remove unbound ^{125}I-apoA-I, 15 mL of DMEM was added, and the plates were immediately incubated at 37°C for 1.4 h, 8 h and 24 h. The medium was concentrated and ^{125}I-apoA-I-containing particles were electrophoretically separated by 2D-PAGGE. As shown in Fig. 6, ^{125}I-apoA-I incubated for 24 h without cells had a pre-β electrophoretic mobility with a molecular diameter of 7.1 nm

(panel A). However, apoA-I-containing particles dissociated from normal stimulated cells at either 1.4 h, 8 h or 24 h exhibited α-electrophoretic mobility with a particle size ranging from 9 to 20 nm (designated α-LpA-I-like particles) (panels, B, C and D, respectively). Both the charge and size of these nascent particles are stable over a 24 h dissociation period. We next examined whether the α-electrophoretic mobility of LpA-I-like particles may be caused by specific phospholipid composition. Cells were first labeled with ^{32}P-orthophosphate, then stimulated and incubated with 10 μg/mL of unlabeled apoA-I for 2 h at 37°C. Dissociated ^{32}P-phospholipidated apoA-I was analyzed by 2D-PAGGE as described above. As shown in Fig. 6 (panels, E, F and G), ^{32}P-phospholipidated apoA-I co-localized with the majority of ^{125}I-α-LpA-I-like particles (panels, B, C and D). We next determined the relative phospholipid composition of α-LpA-I-like particles. The medium containing α-LpA-I-like particles at different time was concentrated, dialyzed and apoA-I-containing particles were immunoprecipitated with an anti-apoA-I antibody. The ^{32}P-labeled phospholipids sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), and phosphatidylinositol (PI) were extracted from immunoprecipitated medium, then separated in triplicate on TLC and quantified by phosphorimager. Percent phospholipid composition of α-LpA-I-like particles was: SM, 16 ± 1%; PC, 51 ± 1%; PE, 15 ± 0.6%; LPC, 4.4 ± 1.3% and PI, 14 ± 0.2%. The ratio of phospholipid species present in α-LpA-I-like particles did not change significantly at either 1.4 h, 8 h or 24 h dissociation period (data not shown).

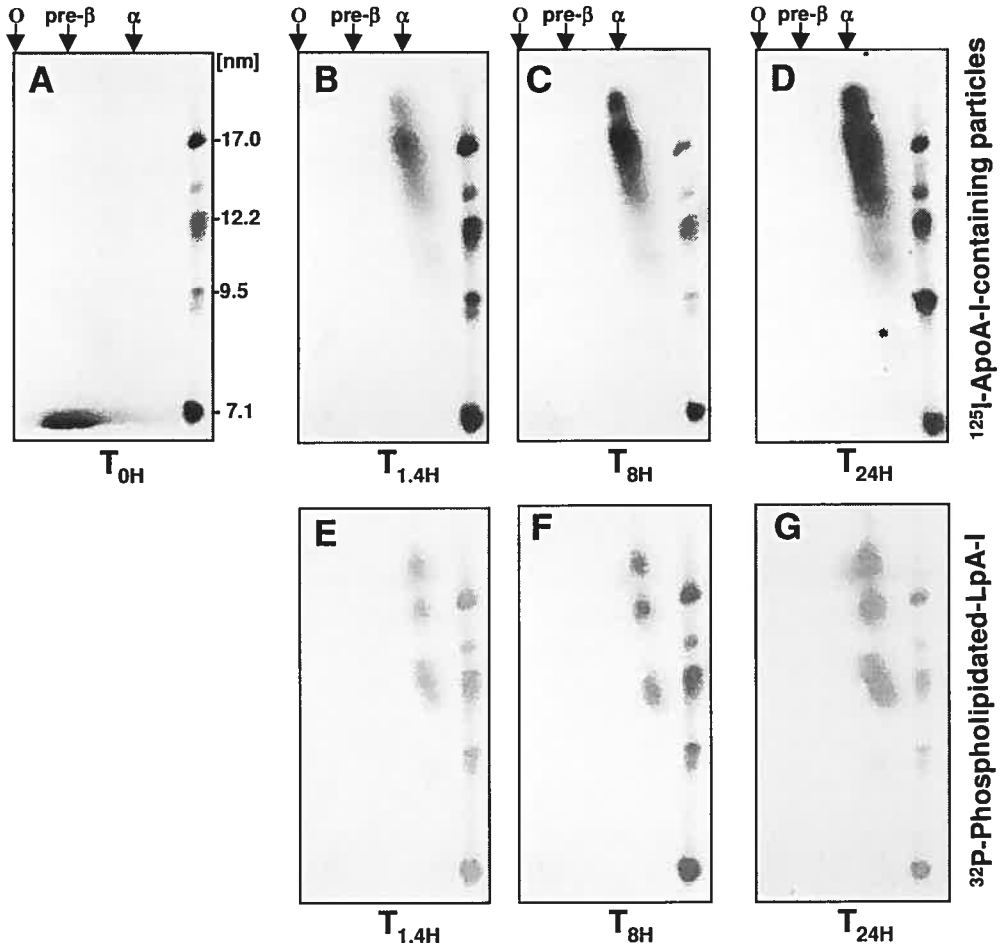


Figure 6. Time course of the formation of apoA-I-containing particles during the dissociation period. Upper panels, stimulated normal cells were incubated with 10 µg/mL ^{125}I-apoA-I for 2 h at 37°C. After washing to remove unbound ^{125}I-apoA-I, 15 mL DMEM was added, and the plate was immediately incubated at 37°C for either 1.4h, 8 h or 24 h. The medium was recovered, concentrated and ^{125}I-apoA-I-containing particles at 1.4 h (***B***), 8 h (***C***) , 24 h (***D***) or ^{125}I-apoA-I incubated in DMEM / BSA for 24 h at 37°C without cells (***A***) were separated by 2D-PAGGE. ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film. **Lower panels,** ^{32}P-orthophasphate-labeled normal cells were stimulated, and then incubated with 10 µg/mL unlabeled apoA-I for 2 h at 37°C. After washing to remove unbound apoA-I, 15 mL DMEM was added, and the plate was immediately incubated at 37°C for either 1.4 h, 8 h or 24 h. The medium was recovered, concentrated, dialyzed and ^{32}P-labeled phospholipids associated with apoA-I-containing particles were analyzed by 2D-PAGGE. ^{32}P-labeled phospholipids associated with apoA-I-containing particles at 1.4 h(***E***), 8 h(***F***) or 24 h (***G***) was directly detected by autoradiography using XAR-2 Kodak film. Molecular size markers are indicated on the right side of each gel. Results shown are representative of two different independent experiments.

DISCUSSION

In view of the importance of ABCA1 in the regulation of plasma HDL cholesterol (1,3,18), we investigated the molecular and physiological mechanisms of ABCA1-dependent apoA-I lipidation in fibroblasts as a model for peripheral cells. Consistently with an earlier study by Remaley *et al.* (16), we show that specific binding parameters of ^{125}I-apoA-I to ABCA1 could be measured in ABCA1-stimulated cells (Fig. 1A). The specificity of ^{125}I-apoA-I binding to ABCA1 was supported by experiments showing that apoA-I forms a complex with ABCA1 in unstimulated cells, and this effect was markedly enhanced in stimulated cells (Fig. 1B). Moreover, increased apoA-I/ABCA1 complex formation was concomitant with increased cellular phospholipid and cholesterol efflux (Fig. 1C). Several groups have reported binding studies with apoA-I conducted at 4°C and the results have been somewhat inconsistent (14,29). Here we demonstrate that the apoA-I cell association showed remarkable temperature dependence, suggesting that apoA-I binding to ABCA1 may be controlled by an energy-dependent process or, alternatively, the change in temperature may alter the lipid conformation in the plasma membrane, which then could affect apoA-I cell association. This result underscores the importance of using physiological temperatures to study apoA-I/ABCA1 interactions.

To gain further insight into the relationships between the conformation/organization of apoA-I within lipidated HDL particles and its interaction with ABCA1, we performed competition assays that clearly showed that plasma preβ_1-LpA-I, reconstituted HDL particles r(LpA-I), and native HDL_3 particles are poor competitors for the binding of ^{125}I-apoA-I to ABCA1 compared to lipid-free apoA-I (Fig. 2A). This experiment indicates an important role for the association of apoA-I with lipids in controlling apoA-I/ABCA1 interactions. Surprisingly, preβ_1-LpA-I, which comprises apoA-I combined with only a small amount of phospholipids (30) had a 4-fold lesser efficiency to interact with ABCA1 relative to lipid-free apoA-I (Fig. 2A). Previous studies established that the lipid composition of preβ_1-LpA-I species as well as the conformation of apoA-I within these particles differ from those of spherical HDL (25,30). Furthermore, preβ_1-LpA-I is proposed to be an initial acceptor of cell-derived cholesterol (30). This supports the idea that preβ_1-LpA-I removes cellular lipid by an aqueous diffusion process rather than an ABCA1-dependent pathway. The physiological relevance of the ABCA1-HDL interaction remained to be determined.

Although evidence have been presented demonstrating molecular interactions between ABCA1 and apoA-I (13,14,16), it remains controversial whether there is a "molecule-to-molecule contact" between apoA-I and ABCA1. Several competing models

have been proposed for this interaction: 1) Burgess *et al.* (31) suggested that phospholipids contained in the extracellular matrix of macrophages act as an initial tether point for apoA-I, bringing it into close proximity to membrane-bound ABCA1; 2) Chambenoit *et al.* (14) have reported that even though ABCA1 expression increases the amount of membrane bound apoA-I, its association with cellular membranes exhibits diffusional properties that are consistent with apoA-I binding to membrane lipids rather than an integral membrane protein. In the present study, experiments have been designed to answer this controversy and evidence was in fact obtained demonstrating that both of these models can not be applied to apoA-I/ABCA1 interactions. Here, we demonstrate that treatment of intact stimulated cells with phospholipases (PC-PLC or SM-ase) affected neither the specific binding of ^{125}I-apoA-I nor apoA-I/ABCA1 cross-linking (Fig. 3A,B). It is likely that apoA-I/ABCA1 interactions are due to a direct protein-protein contact, which is not dependent on the presence of membrane phosphatidylcholine or sphingomyelin. However, it is not excluded that other membrane phospholipids or the phospholipase lipid products may serve to bind the amphiphathic helix of apoA-I to ABCA1. This is consistent with a previous study by Smith *et al.* (32) showing that although ABCA1 expression is associated with an increase in cell surface phosphatidylserine level, the cellular association of apoA-I is not competed by annexin V, a phosphatidylserine binding protein. Moreover, Mendez *et al.* (33) documented that cholesterol and sphingomyelin-rich membrane rafts do not provide lipid for efflux promoted by apolipoproteins through the ABCA1-mediated lipid secretory pathway.

It has been suggested that the correct conformation of ABCA1 thought to be maintained by the ATP hydrolysis action of ABCA1 or its lipid flipping activity (14,19) was necessary for apoA-I binding. Furthermore, recent studies from our laboratory and others have shown that ABCA1 phosphorylation by cAMP/PKA-dependent pathway plays an important role in the apoA-I lipidation reaction (20,34,35), suggesting that lipidation of apoA-I by ABCA1 is an active process. We confirmed and extended this observation by showing that ^{125}I-apoA-I dissociation from ABCA1 was almost completely inhibited at either 4°C or 15°C (Fig. 4A).

The structural requirements of apoA-I lipidation by ABCA1 have not yet been determined. However, in an attempt to understand this process in fibroblasts, we examined apoA-I lipidation reaction in a tissue culture model by monitoring the kinetic parameters of apoA-I dissociation from ABCA1. We initially hypothesized that any specific apoA-I dissociation from ABCA1 would be associated with a significant increase in apoA-I

lipidation state, consistent with the concept that the transfer of phospholipid and cholesterol from the active site of ABCA1 transporter to apoA-I molecule weakens the interaction of apoA-I/ABCA1 and causes dissociation of the lipidated apoA-I product. Our hypothesis is supported by the finding that: 1) specific apoA-I dissociation from ABCA1 is rapid (Fig. 4A); 2) the association of apoA-I with lipids reduces its ability to interact with ABCA1; 3) the lipid translocase activity of ABCA1 generates α-LpA-I-like particles; and 4) ABCA1 did not mediate hydrolysis of apoA-I in fibroblasts. However, chlorpromazine has been shown to block cAMP-mediated cholesterol efflux in macrophages (36), supporting the idea that ABCA1 may be involved in the endocytosis and resecretion of apoA-I in macrophages. More thorough investigations are required to establish definitively a possible role of ABCA1 in the endocytosis of apoA-I in fibroblasts and macrophages.

Of interest, comparison of the dissociation rate constant of apoA-I from ABCA1 and apoA-I-mediated cholesterol efflux showed for the first time that apoA-I dissociation from ABCA1 is rapid ($t_{½}$ =1.4h, Fig. 4A). In contrast, in our stimulated cell culture system, apoA-I-mediated cholesterol efflux reached saturation after a 16 h incubation (Fig. 4B). Previous studies have demonstrated that lipid-free apolipoproteins access both cellular FC and PL during incubations of 4-24 h (37,38), however, others such as Gillotte *et al.*(39) do show saturation in a short time frame. It should be noted that in their study the fibroblasts were not enriched with cholesterol, ABCA1 was not induced and the cells were labeled with a very high amount of ^{3}H-cholesterol and ^{3}H-choline. Our results suggest that each ABCA1 molecule at the cell surface may have multiple lipidation cycles, which may result in the lipidation of many apoA-I molecules by the same ABCA1 molecule. This concept is supported by an elegant study by Tall and co-workers (34) demonstrating that apoA-I/ABCA1 interactions result in the dephosphorylation of the ABCA1 PEST sequence and thereby inhibits calpain degradation leading to an increase of both ABCA1 cell surface expression and activity.

Several laboratories have demonstrated that apoA-I incubated with cells including fibroblasts (37), CHO cells (40), and macrophages (38) was able to recruit phospholipid and cholesterol from the cells to form protein-lipid complexes. Our experiment presented in Fig. 5 shows that the apoA-I-lipid complexes thus formed during apoA-I incubation with stimulated normal cells represent a spectrum of particles with distinct molecular diameters, in contrast, lipid-free apoA-I was unable to form larger particles during its incubation with Q579R mutant cells. The interrelationship between these particles was unclear; however, a time–course analysis of apoA-I-containing particles dissociated form

ABCA1 (Fig. 6) showed nascent apoA-I-phospholipid complexes that exhibited α-electrophoretic mobility with a particle size ranging from 9 to 20 nm (designated α-LpA-I-like particles). The stability of the charge, molecular diameter and phospholipid species content of these nascent particles over a 24 h dissociation period did not support the existence of a clear precursor-product relationship between the various particles and provide strong support for their common origin. It is important to note that the newly formed α-LpA-I-like particles had distinctly different sizes, suggesting that larger particles contained both phospholipids and cholesterol whereas the smallest particles contained only phospholipids and apoA-I (41). Because of the absence of cholesterol acyltransferase activity in the extracellular medium to convert FC to cholesteryl ester, it is most likely that α-LpA-I-like particles are discoidal. Indeed, it was documented that a lipoprotein with a high concentration of phosphatidylinositol could have a high negative charge and consequently an α- electrophoretic mobility (42), consistent with our finding that α-LpA-I-like particles have high content in phosphatidylinositol (14 ± 0.2%).

During the preparation of this manuscript a study by Liu *et al.* (43) reported that incubation of apoA-I with macrophages leads to the formation of more than one type of lipidated apoA-I-containing particles with a molecular diameter of 6 to 16 nm. In addition, this study support the idea that there is a simultaneous release of PL and FC to apoA-I molecules through a membrane microsolubilization process. It is interesting to contrast our results with those reported in that study, which demonstrated that apoA-I-containing particles have a smaller size and an important amount of apoA-I remaining in its lipid-free form. It is possible that the cell species used in the two studies affect ABCA1-dependent lipidation of apoA-I: we used human fibroblasts and Liu *et al.* (43) used J774 macrophages. In addition our cells were loaded directly with FC (20 µg/mL) and were stimulated with 22 (R)-hydroxycholesterol and 9-*cis*-retinoic acid and their cells were loaded with 25 µg/mL acetyl-LDL and induced with cAMP. More importantly, we observed that both the charge and diameter of the newly formed LpA-I-like particles are markedly different from those of lipid-free apoA-I (Fig. 5 and 6). We therefore suggest that our experimental design based on the analysis of LpA-I particles released during the dissociation period might be critical for the study of LpA-I product generated by a specific lipid translocase activity of ABCA1.

Evidence has been presented here demonstrating that only lipid-free apoA-I is able to interact efficiently with ABCA1 *in vitro* (Fig. 2). However, it seemingly paradoxical that lipid free-apoA-I molecules, which are not normally present in significant quantities in plasma

(44), play a similar role *in vivo*. We postulate that lipid-free apoA-I generated during apoA-I-containing particles remodeling cycle (45) are rapidly lipidated by ABCA1 and form α-LpA-I-like particles or, alternatively, may be incorporated in preexisting plasma HDL. Our current results support the first hypothesis. We demonstrate that 50% of specifically bound ^{125}I-apoA-I was rapidly dissociated from ABCA1 at physiological temperatures ($t_{½}$ ~1.4 h) (Fig. 4A). At the same time, the majority of apoA-I-containing particles generated during the dissociation period was shown to be associated with phospholipids having an α-electrophoretic mobility (Fig. 6E). This concept is supported by recent study by Kee *et al.* (46) demonstrating that the electrophoretic mobility of ^{125}I-apoA-I (lipid-free) changed from pre-β to α- electrophoretic mobility only 2 minutes after injection into wild-type rabbits. In addition, the same study documented that hepatic lipase has the capacity to decrease the size of α-migrating HDL, in agreement with earlier work of Barrans *et al.* (45).

Different kinetic models can be proposed to explain the mechanism of apoA-I/ABCA1 interaction. Here, our cell culture system represents a relatively simple model. However, in peripheral tissues and interstitial fluid, many other lipid-free apolipoproteins (e.g. apoE, apoJ, apoA-IV) might compete with apoA-I for ABCA1 binding. Our results show that apoA-I binding to ABCA1 was found to occur in a time and concentration dependent-manner (Fig. 1A). Thus, apoA-I/ABCA1 association can be described as a receptor-ligand interaction or a protein-protein interaction in solution under apparent equilibrium condition (Fig. 7). On the other hand, the lipid translocase activity of ABCA1 transforms lipid-free apoA-I to α-LpA-I-like particle; here ABCA1 seems to act as an enzyme catalyzing the lipidation of the substrate. Although admittedly speculative, we believe that our data support this hybrid model better than either ligand/receptor or substrate/enzyme model. The important finding that the interaction of lipid-free apoA-I with ABCA1 generates only α-LpA-I-like particles might help to explain why lipid-free apoA-I is found in trace amounts in human plasma. Indeed, following the release of lipid-free apoA-I by the action of hepatic lipase on HDL_2 and a possible involvement of SR-BI in this process (47), lipid-free apoA-I molecules might be very rapidly lipidated by ABCA1 and transformed into α-LpA-I-like particles (Fig. 7).

The results presented in this study provide a biochemical basis for a cellular apoA-I lipidation pathway that involves ABCA1 protein in peripheral cells. This process plays *in vivo* a key functional role in the biogenesis of nascent HDL particles.

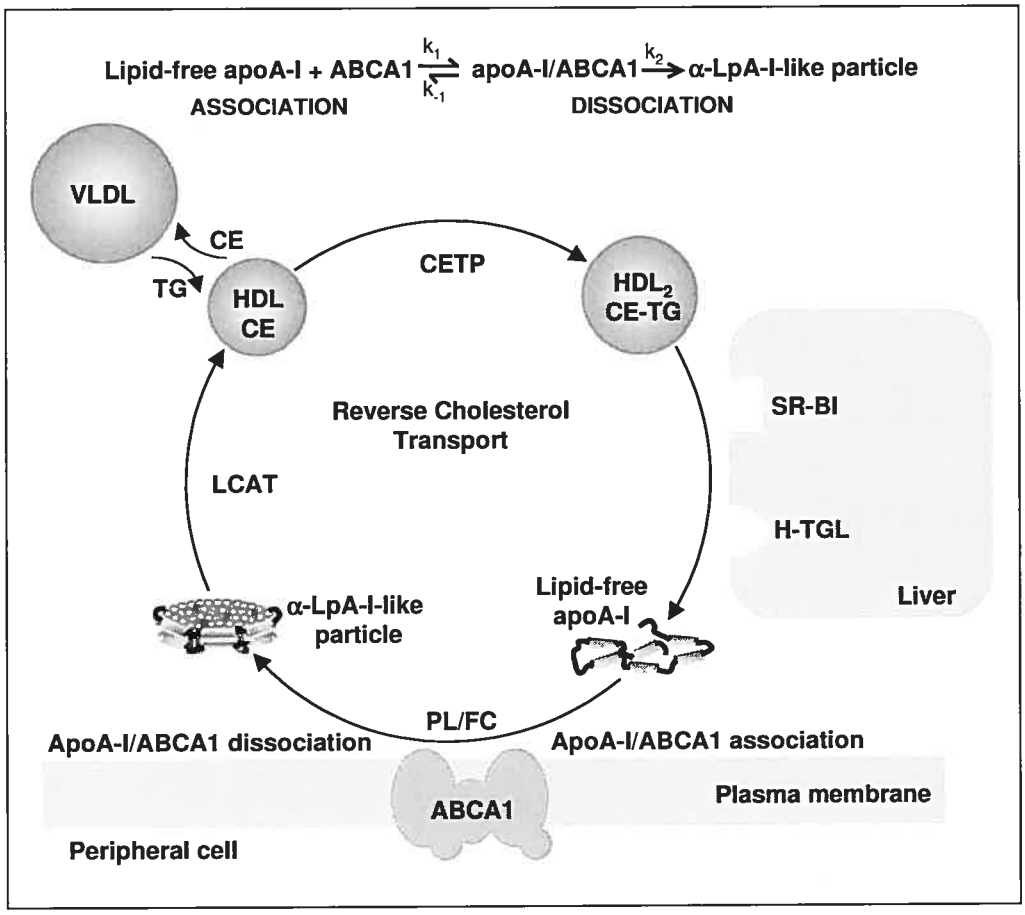


Figure 7. A proposed model of free apoA-I lipidation by ABCA1 in peripheral cells. Cholesteryl ester-rich HDL_2 gain triacylglycerols from VLDL under the action of CETP. HDL_2 undergo lipolysis by the hepatic lipase (H-TGL) and a possible involvement of the SR-BI receptor generating lipid-free apoA-I, which can be rapidly lipidated by ABCA1 and form α-LpA-I-like particles. Continuous action of LCAT contributes to the maturation of α-LpA-I-like particles to form cholesteryl ester-enriched HDL. A model of apoA-I lipidation by ABCA1 can be proposed assuming that 1) initial binding of apoA-I to ABCA1 is irreversible or slowly reversible ($k_1 >> k_{-1}$); 2) lipidated apoA-I (α-LpA-I-like particles) dissociate rapidly (k_2) from ABCA1 without any detectable reassociation; and 3) this system contains no other apolipoprotein that could compete for the binding of lipid-free apoA-I to ABCA1. PL, phospholipids; FC, free cholesterol; TG, triglycerides; CE, cholesteryl esters.

ACKNOWLEDGMENTS

This work was supported by grants MOP 15042 from the Canadian Institutes of Health Research (CIHR), and the Heart and Stroke Foundation of Canada. M. Denis was supported by a personnel award from the Heart and Stroke Foundation of Canada. J. Genest holds the McGill University-Novartis Chair in Cardiology.

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Chapitre V

Article #3 *

1. Contexte de travail

Même s'il a été démontré que d'autres transporteurs ABC forment des dimères et des tétramères, rien n'était connu sur la structure quaternaire de l'ABCA1. Le fait que l'interaction apoA-I/ABCA1 génère plusieurs tailles de particules migrant en position alpha a suggéré que toutes ces particules n'ont pas la même composition en phospholipides, en cholestérol et en apoA-I. Aussi, il était probable que l'ABCA1 favorise la dimérisation de l'apoA-I en lui transférant des lipides. Ainsi, il a été postulé que soit une molécule d'ABCA1 lie deux molécules d'apoA-I, soit deux molécules d'ABCA1 contigües servent d'ancrage à deux molécules d'apoA-I.

C'est dans ***le but de déterminer la structure fonctionnelle de l'ABCA1*** que le travail a été entrepris.

Un indice a permis d'entreprendre le travail avec la confiance d'obtenir des résultats significatifs: sur un gel de polyacrylamide en conditions dénaturantes et réductrices, une bande de poids moléculaire supérieur à 250 kDa et immunoréactive avec un anticorps anti-ABCA1 était souvent détectée. En effet, il est d'usage de ne pas bouillir les échantillons avant de charger sur gel, car cela rend l'entrée d'ABCA1 dans le gel difficile. Ainsi, il en résulte une hydrolyse incomplète des ponts disulfures intermoléculaires, ce qui explique la bande observée à un poids moléculaire supérieur.

Dans l'article suivant,

Maxime Denis a fait la majeure partie du travail et a co-écrit le manuscrit
Bassam Haidar a fait la figure 4
Michel Marcil a dessiné la figure 6
Michel Bouvier a participé à la supervision
Larbi Krimbou a co-écrit le manuscrit et a aidé à faire les gels
Jacques Genest a supervisé le travail

*** Note de l'auteur :** Cet article mis en page sous forme "pdf" est disponible en annexe

2. Article:

Characterization of Oligomeric Human ATP-binding Cassette Transporter A1
POTENTIAL IMPLICATIONS FOR DETERMINING THE STRUCTURE OF NASCENT HDL PARTICLES

(Accepted for publication in *The Journal of Biological Chemistry)*
(Published on-line on July 26th 2004 as "Paper in Press" manuscript #M406881200)

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Running title: Characterization of oligomeric ABCA1 transporter

ABSTRACT

The oligomeric structure of ABCA1 transporter and its function related to the biogenesis of nascent apoA-I-containing particles (LpA-I) were investigated. Using n-dodecylmaltoside and perfluoro-octanoic acid combined with non-denaturing gel, the majority of ABCA1 was found as a tetramer in ABCA1-induced human fibroblasts. Furthermore, using chemical cross-linking and SDS-PAGE, ABCA1 dimers but not the tetramers were found covalently linked. Oligomeric ABCA1 was present in isolated plasma membranes as well as in intracellular compartments. Interestingly, apoA-I was found to be associated with both dimeric and tetrameric, but not monomeric forms of ABCA1. Neither apoA-I nor lipid molecules did affect ABCA1 oligomerization. Immunoprecipitation analysis showed that oligomeric ABCA1 did not contain other associated proteins. We next investigated the relationship between the oligomeric ABCA1 complex and the structure of LpA-I. Lipid-free apoA-I incubated with normal cells generated LpA-I with diameters between 9.5 to 20 nm. Subsequent isolation of LpA-I followed by cross-linking revealed the presence of 4 and 8 apoA-I molecules per particle, whereas apoA-I incubated with ABCA1 mutant (Q597R) cells was unable to form such particles and remained in the monomeric form. These results demonstrate that: 1) ABCA1 exists as oligomeric complex; and 2) ABCA1 oligomerization was independent of apoA-I binding and lipid molecules. The findings that the majority of ABCA1 exists as a tetramer which binds apoA-I, together with the observation that LpA-I contains at least 4 molecules of apoA-I per particle, support the concept that the homotetrameric ABCA1 complex constitutes the minimum functional unit required for the biogenesis of HDL particles.

Keywords: ABCA1, HDL, LpA-I, efflux

ABBREVIATIONS

2D-PAGGE, two-dimensional polyacrylamide non-denaturing gradient gel electrophoresis; ABCA1, ATP binding cassette AI; apo, apolipoprotein; BSA, Bovine serum albumin; DSP, dithiobis(succinimidylpropionate); DTNB, 5,5-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; FHD, Familial HDL deficiency; HDL, high density lipoprotein; ICC, intracellular compartments; LpA-I, nascent apoA-I-containing particle; PL, phospholipids; PM, plasma membrane; PFO, perfluoro-octanoic acid; RCT, reverse cholesterol transport; TD, Tangier disease; UC, unesterified cholesterol.

INTRODUCTION

ABCA1 is a 240-kDa protein belonging to a large family of conserved transmembrane proteins that transport a wide variety of substrates, including lipids, ions, amino acids, peptides, sugars, vitamins, steroid hormones, and drugs across cell membranes (1). ABC transporters have been associated with many diseases such as drug-resistant cancer (2), diabetes (3), and cystic fibrosis (4).

Apolipoprotein (apo) A-I binding to the extracellular domain of ABCA1 results in the activation of apoA-I lipidation, a key step in reverse cholesterol transport (RCT) process, one of the major mechanisms by which high density lipoprotein (HDL) may protect against atherosclerotic vascular disease (5-7). The molecular interaction of apoA-I with ABCA1 promotes cholesterol and phospholipid efflux from peripheral cells and macrophages. However, Brewer and colleagues (8) recently reported that hepatic ABCA1 is a key protein for the formation and maintenance of plasma HDL levels. Moreover, the importance of ABCA1 in the lipidation of apoA-I is highlighted by the finding that over 50 mutations in the ABCA1 gene have been associated with a variety of clinically distinct HDL-deficiency diseases including Tangier Disease (TD) and Familial HDL Deficiency (FHD) (9-11). These patients are characterized by excess cholesterol accumulation in macrophages, low plasma HDL levels, and increased risk of coronary artery atherosclerosis (12).

ABC transporters typically consist of two multi-spanning membrane domains (MSDs) that serve as a pathway for the translocation of substrates across membranes and two ATP binding cassettes or nucleotide binding domains (NBDs) that provide the energy for substrate transport (13,14). These domains are found either on a single long polypeptide chain as in the case of CFTR and the multidrug resistance proteins, P-glycoprotein and MRP1, or as a complex of two identical or similar "half-molecule" subunits each having an MSD and an NBD, as found in the TAP1/TAP2 ABC transporters associated with peptide antigen processing. ABCA1 belongs to the first category, because it consists of a single polypeptide comprised of two arranged halves. Each half contains an MSD followed by a cytoplasmic NBD. A distinguishing feature of ABCA1 is the presence of a large exocytoplasmic domain that connects the first transmembrane segment to the MSD in each half of the protein (15).

Although the ABCA1 molecule is well characterized, very little is known concerning its quaternary structure and its functional properties related to the formation of nascent apoA-I-containing particles. To date, no studies have directly assessed the multimeric structure of human ABCA1. It was therefore the aim of the present study to provide evidence for the

existence of oligomeric ABCA1 complex, to demonstrate how ABCA1 forms could be affected by apoA-I, or lipid molecules, and to examine the impact of the oligomeric ABCA1 complex on the structure of nascent apoA-I-containing particles in a cell culture model.

EXPERIMENTAL PROCEDURES

Patients selection — For the present study, we selected fibroblasts from 3 normal control subjects and 1 patient with TD (homozygous for Q597R at the ABCA1 gene). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Centre. Separate consent forms for blood sampling; DNA isolation and skin biopsy were provided.

Cell culture — Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearm of patients and healthy control subjects and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum (FBS). Human GFP-ABCA1 expressing CHO cells were generously provided by Dr. Sean Davidson, Department of Pathology and Laboratory Medecine, University of Cincinnati, and were characterized and cultured as described previously (16).

Human plasma apoA-I and apoE3 — Purified plasma apoA-I (Biodesign) was resolubilized in 4M guanidine-HCl and dialyzed extensively against PBS buffer and used within 24 h. ApoA-I was iodinated with ^{125}Iodine by IODO-GEN® (Pierce) to a specific activity of 800-2500 cpm/ng apoA-I. Purified human plasma apoE3 was a gift from Dr. Karl H. Weisgraber (Gladstone Institutes of Cardiovascular Disease, San Francisco, CA).

Solubilization of cell proteins by n-dodecylmaltoside and perfluoro-octanoic acid (PFO) — Normal fibroblasts in 100-mm diameter dishes were stimulated with 2.5 µg/mL 22 (R)-hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h. Cells were then lysed at 4°C with PBS containing 0.5% n-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble materials. In separate experiments, cells were lysed with 0.8% perfluoro-octanoic acid (PFO) as described by Ramjeesingh and colleagues (17). After solubilization of cell proteins and centrifugation at 11,000 *x g*, 4°C, for 10 min, the supernatants were treated or not with 50 mM dithiothreitol (DTT) for 30 min at 37°C and then the samples were separated by non-denaturing gradient gel electrophoresis (3-15%) as previously described (18).

Chemical cross-linking and immunoprecipitation (IP) analysis — Chemical cross-linking was performed as described by Tall and colleagues (19) with a minor modification. Fibroblasts were grown to confluence in 100-mm diameter dishes and then stimulated or not with 2.5 µg/mL 22-(R) hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h in DMEM/BSA. Cells were incubated in the presence or absence of 10 µg/mL of ^{125}I-apoA-I in DMEM/BSA for 2 h at 37°C. Cells were then placed on ice for 15 min and washed three

times with PBS. Dithiobis(succinimidylpropionate) (DSP, cross-linker agent) was dissolved immediately before use in dimethyl sulfoxide (DMSO) and diluted to 500 µM in PBS. Six mL of DSP solution was added in each well. Cells were then incubated at room temperature for 30 min; the medium was removed, and the cells were washed twice with PBS. Cells were lysed at 4°C with 20 mM Tris 5 mM EDTA / 5mM EGTA; pH 7.5) containing 0.5% n-dodecylmaltoside and the suspension was allowed to stand for 10 min at 4°C in presence of a protease inhibitor cocktail (Roche Diagnostics). Either apoA-I/ABCA1 complex or ABCA1 alone was immunoprecipitated with an affinity purified polyclonal anti-ABCA1 antibody (Novus Biologicals) as previously described (20,21). After SDS-gel electrophoresis ABCA1 was detected by an affinity-purified human anti-ABCA1 antibody. The presence of labeled ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography using XAR-2 Kodak film.

Isolation of nascent LpA-I particles and cross-linking with DSP — ^{125}I-apoA-I (10 µg/mL) was incubated in DMEM for 24 h at 37°C with either stimulated normal or Q597R cells. LpA-I particles were isolated using ultrafiltration (spiral ultrafiltration cartridge, MWCO 100,000, Amicon) to discard any lipid-free apoA-I. LpA-I particles were further dialyzed using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I. Cross-linking of isolated LpA-I was performed as described by Davidson and colleagues (22) with slight modifications. Either 15 µg of isolated LpA-I generated by normal cells, apoA-I incubated with Q597R cells or lipid-free apoA-I incubated without cells were adjusted to 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, with a final protein concentration of 1 µg/µL. Immediately before an experiment, 1 mg of DSP was dissolved in 1000 µL of DMSO to a final concentration of 1 µg/µL. The dissolved DSP was added to the reaction mixture for 10 DSP to 1 apoA-I molar ratio on ice. The reaction was incubated at 4°C for 24 h with periodic vortexing. The reaction was quenched by adding 1M Tris, pH 7.8, to a final Tris concentration of 100 mM. To rule out the possibility that cross-linking conditions might affect the number of apoA-I molecules per particle, the molar ratio of DSP to apoA-I was varied from 5/1 to 20/1 and incubations were conducted at different temperatures (4°C, 37°C and room temperature).

Cell surface biotinylation — Performed as described previously (23) with slight modifications. Confluent cells were stimulated and then cross-linked with DSP as described above. Surface proteins were biotinylated with 500 µg/mL NHS-SS-biotin (Pierce) for 30 min at 4°C. Cells were then washed with ice cold quench buffer [1M Tris-HCl (pH 7.5)] and twice with ice cold PBS. The cells were lysed at 4°C with 20 mM Tris 5

mM EDTA / 5mM EGTA (pH 7.5) containing 0.5% n-dodecylmaltoside in presence of a protease inhibitor cocktail and then homogenized with 40 strokes in a tight fitting dounce homogenizer. After centrifugation at 1000 *x g*, 4°C, for 10 min to remove unbroken cells and nuclei; 200 µg of protein from the supernatant was added to 30 µL streptavidin-sepharose (Amersham) and incubated overnight on a platform mixer at 4°C. The beads was pelleted and washed 3 times with lysis buffer. Cross-linking, SDS-PAGE and detection of ABCA1 were performed as described above.

Metabolic labeling and immunoprecipitation of ABCA1 — Metabolic labeling of ABCA1 was performed as described by Oram and colleagues (24). Briefly, either stimulated or unstimulated normal cells were labeled with 150 µCi/mL [^{35}S]-methionine for 4 h. Cells were then lysed at 4°C with lysis buffer containing 0.5% n-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble materials. The supernatants were immunoprecipitated with an anti-ABCA1 antibody. Immunoprecipitated samples were separated on 4-22.5% SDS-PAGE and [^{35}S]-labeled ABCA1 was directly detected by autoradiography.

Separation of lipoproteins by two-dimensional non-denaturing gradient gel electrophoresis (2D-PAGGE) — ApoA-I-containing particles were separated by 2D-PAGGE, as previously described (18). Briefly, samples (30-100 µL) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4°C) and in the second dimension (according to the size) by 5-23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4°C). Iodinated high molecular weight protein mixture (7.1 nm to 17.0 nm, Amersham) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4°C) onto nitrocellulose membranes (Hybond ECL, Amersham). ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film.

RESULTS

In the present study, we have examined the multimeric status of human ABCA1 transporter in normal intact fibroblasts stimulated with 22 (R)-hydroxycholesterol and 9-*cis*-retinoic acid (22OH/9CRA) using both n-dodecylmaltoside and perfluoro-octanoic acid (PFO) combined with non-denaturing gel electrophoresis. These detergents at appropriate concentrations do not break the non-covalent interactions between protein subunits of an oligomer allowing determination of the oligomeric structure of ABCA1 complex. As shown in Fig. 1A, detection of ABCA1 by anti-ABCA1 antibody, after separation of total cell lysate solubilized by a non-ionic detergent n-dodecylmaltoside (0.5%) on non-denaturing gel (3-15%), revealed both a major and minor bands. The major band migrated as a ≈950 kDa complex, consistent with molecular mass of tetramers. The minor band migrated as a larger complex, possibly an oligomer higher than tetramer, whereas the band with apparent molecular mass of ≈550 kDa is likely a dimer. On the other hand, using DTT as a reducing agent we observed that all the oligomeric forms were reduced to the monomeric form with molecular mass of ≈250 kDa reported for complex glycosylated ABCA1 protein as estimated using SDS-PAGE (21). Similar results were also observed with a mild ionic detergent PFO (Fig. 1B), suggesting that the oligomeric ABCA1 observed was not due to the use of a specific detergent. Interestingly, we did not detect any ABCA1 with the size of a monomer on non-denaturing gel in the absence of DTT.

To determine further whether the oligomeric ABCA1 complex exists in living cells, chemical cross-linking was performed as described by Tall and colleagues (19). As shown in Fig. 2A, using SDS-PAGE under non-reducing conditions, ABCA1 migrated at either the monomeric and dimeric molecular masses (≈250 and ≈500 kDa, respectively) whereas the monomer was predominant in the presence of DTT, indicative of disulfide bond contribution in dimer formation. On the other hand, a chemical cross-linker, DSP, was applied to the surface of intact normal fibroblasts to assess the quaternary structure of ABCA1. We found that immunoreactive ABCA1 in cells treated with DSP migrated primarily as a monomer (≈250 kDa), dimers (~ 500 KDa) or as a larger complex with a mass greater than that predicted for either a monomer or a dimer (Fig. 2B). This larger

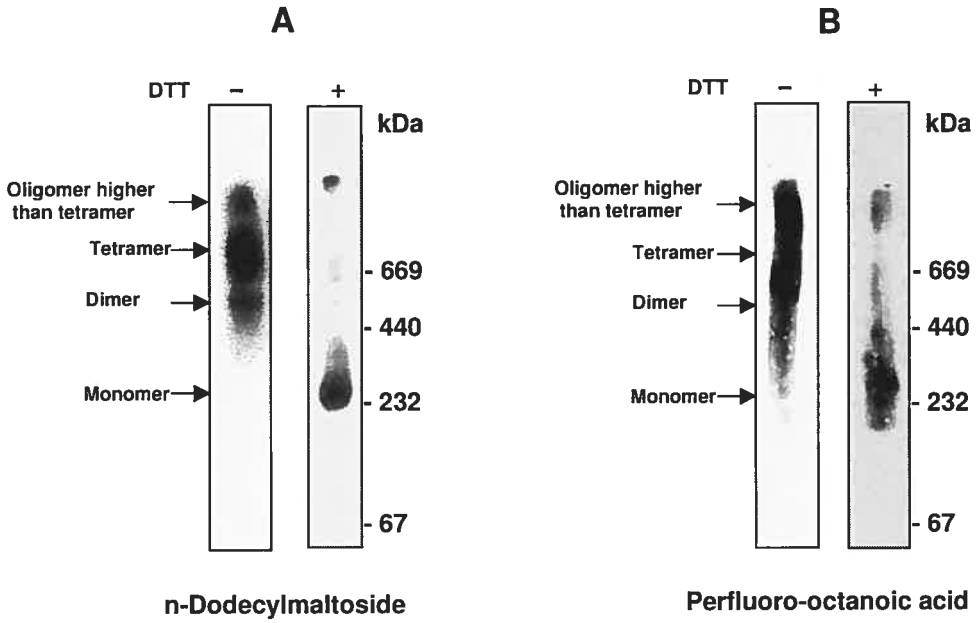


Figure 1. Analysis of oligomeric ABCA1 complex by non-denaturing gradient gel electrophoresis (PAGGE). ***A***, normal fibroblasts in 100-mm diameter dishes were stimulated with 2.5 µg/mL 22 (R)-hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h. Cells were then lysed at 4°C with PBS containing 0.5% n-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble material. The supernatants were treated or not with 50 mM DTT for 30 min at 37°C and then separated by non-denaturing PAGE. After electrophoresis ABCA1 was detected with an affinity-purified polyclonal anti-ABCA1 antibody. ***B***, stimulated fibroblasts were lysed at 4°C with PBS containing 0.8% perfluoro-octanoic acid. The supernatants were treated or not with 50 mM DTT for 30 min at 37°C and then separated by PAGGE. ABCA1 complex was detected as described in A. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa) were used as markers.

band is likely a tetramer. DTT reduced all the oligomeric forms to the monomeric ABCA1. To assess further the subcellular distribution of oligomeric ABCA1, we employed surface biotinylation to isolate ABCA1 associated with plasma membrane (PM). Cross-linking of intact normal stimulated fibroblasts followed by biotinylation and detection of ABCA1 on SDS-PAGE showed three bands associated with PM, corresponding to monomeric, dimeric and tetrameric ABCA1 (Fig. 2C, left panel). The three ABCA1 forms were also detected in the intracellular compartments (ICC) (Fig. 2C, right panel).

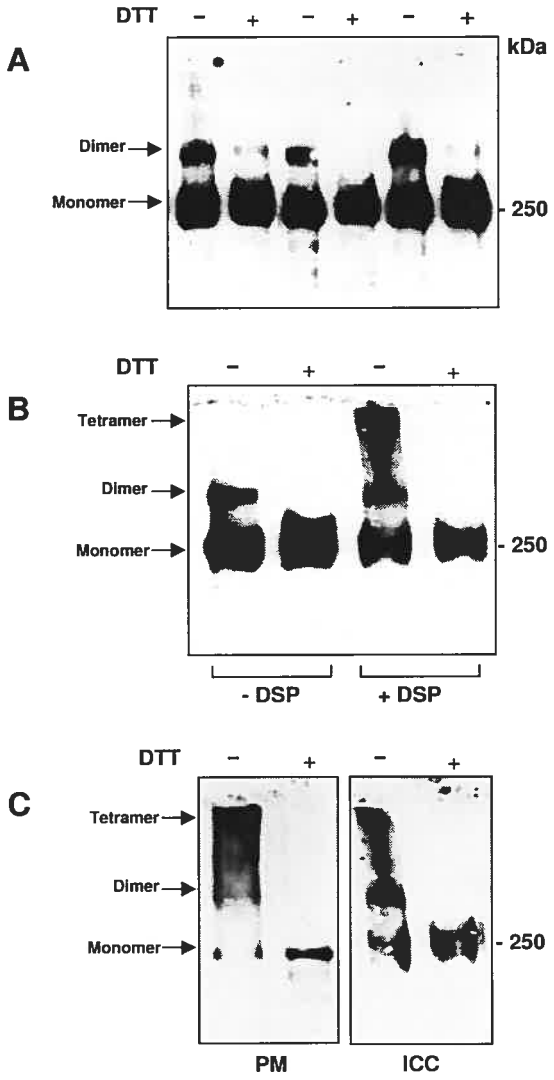


Figure 2. Chemical cross-linking of ABCA1 in intact fibroblasts and the cellular localization of the oligomeric ABCA1 complex. ***A***, normal fibroblasts in 100-mm diameter dishes were stimulated with 2.5 μg/mL 22 (R)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Cells were then lysed at 4°C with lysis buffer containing 0.5% n-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble material. The supernatants were treated or not with 50 mM DTT for 30 min at 37°C and then separated by SDS-PAGE (4-22.5%) in triplicate. ABCA1 was detected as in Fig. 1. ***B,*** stimulated cells were cross-linked or not with 500 μM DSP and the cells were lysed and reduced or not with DTT as described in A. After electrophoresis on SDS-PAGE, ABCA1 was detected by an anti-ABCA1 antibody. ***C,*** stimulated cells were cross-linked with DSP and surface biotinylation was employed to isolate ABCA1 associated with plasma membrane (PM) as described under "Experimental Procedures". ABCA1 associated with intracellular compartments (ICC) was immunoprecipitated by an anti-ABCA1 antibody. Samples containing either PM or ICC were reduced or not with DTT and then separated by SDS-PAGE. ABCA1 was detected by an anti-ABCA1 antibody.

Having determined that oligomeric ABCA1 complex was present in normal human fibroblasts treated with 22OH/9CRA, the question was posed whether ABCA1 induction in fibroblasts may cause self-association events that are non-physiological. We next examined the presence of oligomeric ABCA1 complex in CHO cells overexpressing human ABCA1. We found that the monomeric and dimeric ABCA1 forms were present on SDS-PAGE under non-reducing conditions, whereas tetrameric ABCA1 was detected in the presence of the cross-linker reagent (DSP) (data not shown). In order to verify that the oligomerization of ABCA1 is not due to oxidation during cell lysis and membrane preparation, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was used as agent that inhibited disulfide bond formation and the dimerization (25). We found that the absence or presence of DTNB did not prevent the dimerization of ABCA1.

Because the physical interactions between apoA-I and ABCA1 have been proposed to be important in the lipidation of apoA-I (26), the question was raised whether lipid-free apoA-I could bind to different ABCA1 forms. Stimulated cells were incubated or not with 10 µg/mL of ^{125}I-apoA-I for 2 h at 37°C, and then cross-linking with DSP were performed. As shown in Fig. 3B, ^{125}I-apoA-I associated with ABCA1 co-localized with both dimeric and tetrameric ABCA1 complex (Fig. 3A), whereas ^{125}I-apoA-I was not found associated with monomeric ABCA1 (Fig. 3). Moreover, the absence or presence of apoA-I did not affect ABCA1 oligomerization (Fig. 2B and Fig. 3A, respectively). In order to verify that ABCA1 oligomerization was not dependent on the presence of lipids, cell lysates were delipidated or not (3 times) with ethanol-ether 3:1, and then cross-linking were performed. Removal of total cellular lipids did not prevent ABCA1 oligomerization (data not shown).

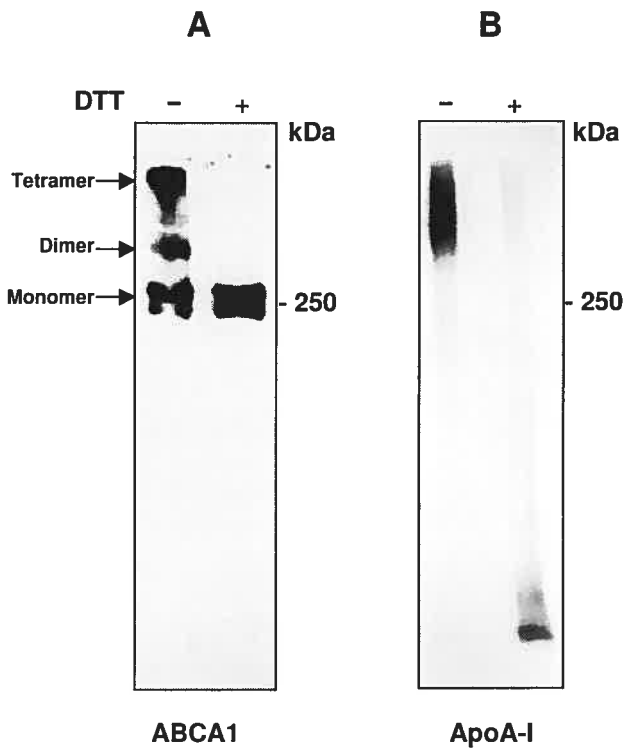


Figure 3. Association of apoA-I with the oligomeric ABCA1 complex. ***A,*** stimulated normal cells were incubated with 10 µg/mL of ^{125}I-apoA-I for 2 h at 37°C. Cross-linking with DSP was performed as described above. Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 µg total protein) were incubated with 10 µL of affinity-purified human anti-ABCA1 antibody for 20 h at 4°C, followed by addition of Protein A bound to sepharose (30 µL). Immunoprecipitated samples were reduced or not with 50 mM DTT for 30 min at 37°C and then separated on 4-22.5% SDS-PAGE. ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography. The ABCA1 protein was detected by an anti-ABCA1 antibody.

To determine whether the oligomeric ABCA1 is a homo- or hetero-oligomer, either stimulated or unstimulated normal fibroblasts were labeled with [^{35}S]-methionine and then ^{35}S-labeled ABCA1 was immunoprecipitated with an anti-ABCA1 antibody. As shown in Fig. 4, the human anti-ABCA1 antibody specifically precipitated no other proteins except human ABCA1. Although it cannot be ruled out that other proteins co-migrate with ABCA1 on SDS-PAGE, the low amount of detectable ^{35}S-labeled material in unstimulated cells did not support this possibility.

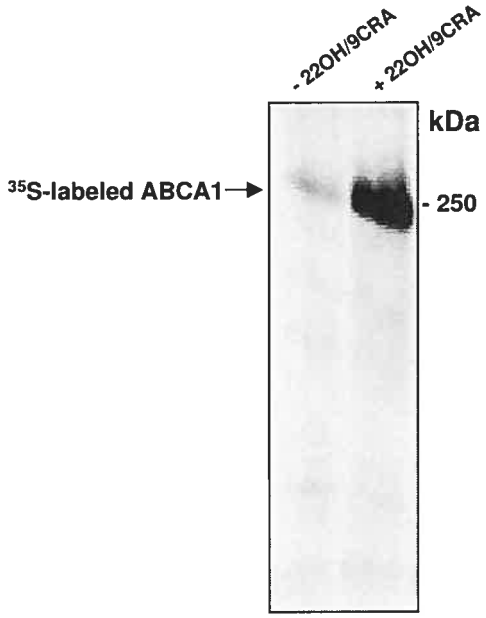
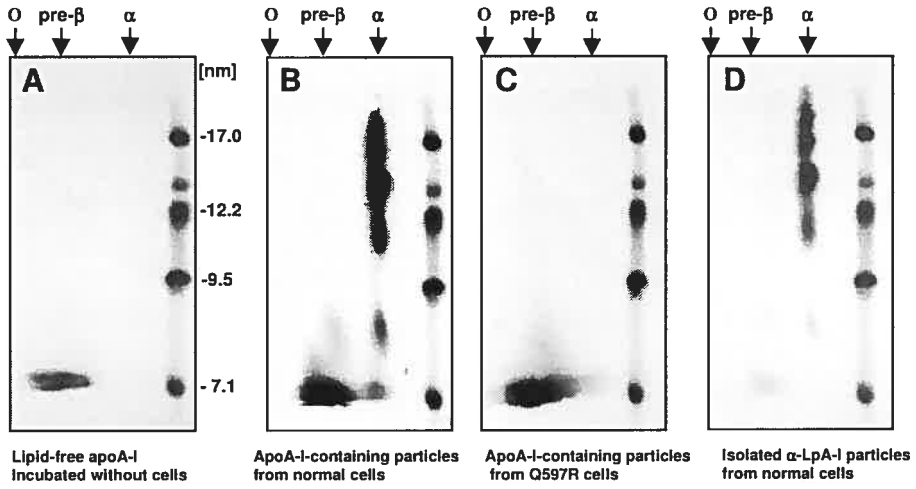


Figure 4. Immunoprecipitation of ^{35}S-labeled ABCA1. Either stimulated or unstimulated normal cells were labeled with 150 μCi/mL [^{35}S]-methionine for 6 h as described under "Experimental Procedures". Cells were then lysed at 4°C with lysis buffer in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble material. The supernatants were immunoprecipitated with an anti-ABCA1 antibody. Immunoprecipitated samples were separated on 4-22.5% SDS-PAGE and [^{35}S]-labeled ABCA1 was directly detected by autoradiography.

To determine the relationship between oligomeric ABCA1 complex and the structural properties of nascent apoA-I-containing particles in our cell culture model, stimulated cells from either normal or from TD (Q597R) subjects in 100-mm diameter dishes were incubated with 10 μg/mL of ^{125}I-apoA-I in 6 mL of DMEM for 24 h at 37°C. The medium was concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 10,000, Amicon) and ^{125}I-apoA-I-containing particles were electrophoretically separated by 2D-PAGGE. As shown in Fig. 5, upper panels, apoA-I-containing particles generated by stimulated normal cells exhibited α-electrophoretic mobility with a particle diameter ranging from 9.5 to 20 nm (Panel B). In contrast, lipid-free apoA-I incubated with stimulated ABCA1 mutant (Q597R) cells was unable to form such particles (Panel C), which had a molecular diameter and charge similar to the lipid-free apoA-I incubated in the same conditions without cells (Panel A). We next isolated LpA-I particles using ultrafiltration (spiral ultrafiltration cartridge, MWCO 100,000, Amicon) to discard any lipid-free apoA-I. LpA-I particles were further dialyzed using a dialysis membrane with a MWCO of 50,000 to remove any remaining

lipid-free apoA-I. As shown in Panel D, isolated LpA-I particles did not contain any significant amount of lipid-free apoA-I.

To further characterize the structure of LpA-I particles generated by ABCA1, isolated LpA-I particles formed by oligomeric ABCA1 complex were incubated with cross-linking reagent dithiobis (succinimidylproprionate) (DSP), a homobifunctional cross-linker that interacts with the -amine group on the side-chain of lysine residues. The cross-linking can occur both intra- and intermolecularly, but only between Lys residues within the reagent's spacer arm length of 12 Å (27). This homobifunctional amine specific cross-linker also possesses a cleavable disulfide bond. Either lipid-free apoA-I incubated without cells or lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells were used as controls. As shown in Fig. 5, lower panel, we consistently observed that LpA-I particles generated by stimulated normal cells contained either 4 or 8 molecules of apoA-I per particle (≈100 and 200 kDa, respectively), which could mostly be reduced back to a monomer by cleavage of the cross-link with DTT. In contrast, both lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells and lipid-free apoA-I incubated without cells remained in the monomeric form following cross-linking. To rule out the possibility that cross-linking conditions or iodination of apoA-I might affect the number of apoA-I molecules per particle, the molar ratio of DSP to apoA-I was varied from 5/1 to 20/1 and incubations were conducted at different temperatures (4°C, 37°C and room temperature). On the other hand, unlabeled apoA-I was used in some experiments. We found that neither the amount of the cross-linker nor the iodination of apoA-I affected significantly the number of apoA-I molecules per particle (data not shown).



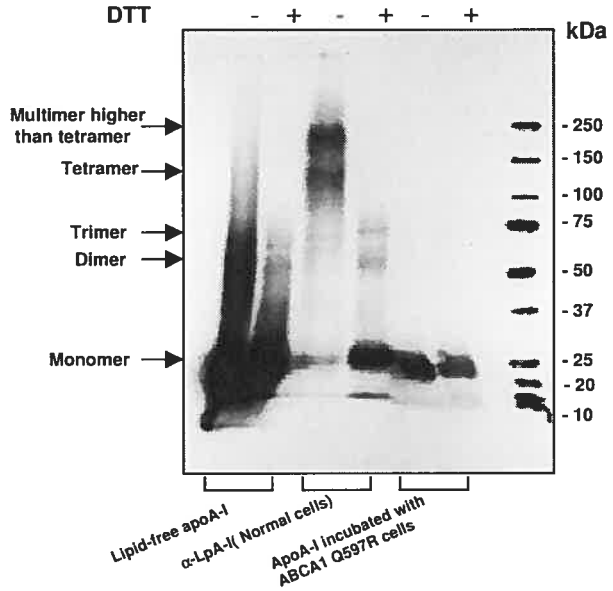


Figure 5. Characterization of nascent LpA-I particles generated by the oligomeric ABCA1 complex. ***Upper panels,*** ^{125}I-apoA-I (10 µg/mL) was incubated in DMEM for 24 h at 37°C without cells (***A***) or with both stimulated normal and Q597R cells (***B and C***, respectively) for 24 h at 37°C. Samples were separated by 2D-PAGGE. We next isolated LpA-I particles using ultrafiltration (spiral ultrafiltration cartridge, MWCO 100,000, Amicon) to discard any lipid-free apoA-I. LpA-I particles were further dialyzed using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I (***D***). ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film. Molecular size markers are indicated on the right side of each gel. ***Lower panel,*** either isolated LpA-I generated by normal cells, apoA-I incubated with Q597R cells or lipid-free apoA-I incubated without cells were treated with DSP as described under "Experimental Procedures". The samples were reduced or not with 50 mM DTT for 60 min at 37°C and then separated on 8-27% SDS-PAGE. ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film. Molecular markers are indicated on the right side of the gel. Results shown are representative of four different independent experiments.

DISCUSSION

The lipid translocase activity of ABCA1 transporter has been implicated in important functions, including the regulation of intracellular lipid trafficking and the lipidation of lipid-poor apolipoproteins to form nascent HDL-particles (20,28,29). It is key that we understand the functional properties of this protein and structural basis for its activity. For the first time, we present evidence that a majority of human ABCA1 exists in intact fibroblasts as a homo-tetramer with a possible higher order of oligomerization (Fig. 1). Similar results were also observed with CHO cells overexpressing human ABCA1, suggesting that the oligomeric ABCA1 complex observed was not due to the use of specific cell types. Interestingly, the absence of ABCA1 monomer as assessed by non-denaturing gel electrophoresis (Fig. 1), suggests that the oligomeric state could even be an essential prerequisite for its sorting, in the trans-Golgi-network and to secretory vesicles. This is consistent with previous studies demonstrating that other ABC transporters such as CFTR, MRP1, or ABCG2 function as either dimers or tetramers (17,30,31).

Although the molecular mechanism of apoA-I binding to oligomeric ABCA1 has not been elucidated, the present study shows that lipid-free apoA-I binds to both dimeric and tetrameric ABCA1 complex (Fig. 3). We believe that these structures are physiologically relevant, it is likely that tetrameric ABCA1 complex constitutes the minimum functional structure required for the apoA-I lipidation process. However, it is possible that the dimeric ABCA1 is a functional lipid transporter and that other oligomeric ABCA1 complex function only as a regulator for the level of a dimeric form, our observation that only a minor proportion of oligomeric ABCA1 exists as a dimer in living cells (Fig. 1A) did not support such a mechanism.

The proposed mechanism of tetrameric ABCA1 complex as the minimum functional unit required for the lipidation of apoA-I was further strengthened by our results demonstrating that nascent apoA-I-containing particles generated by the lipid translocase activity of ABCA1 contain either 4 or 8 molecules of apoA-I per particle. Thus, we provide further evidence for a functional link between oligomeric ABCA1 transporter and the multimeric structure of nascent apoA-I-containing particles. We postulate that functional oligomeric ABCA1 complex is required for the lipid transfer and the assembly of multiple molecules of apoA-I on the same particle. Our current results support this hypothesis. We

demonstrate that lipid free-apoA-I incubated with ABCA1 mutant (Q597R) cells remained in the monomeric form (Fig. 5, lower panel). Furthermore, we have reported that lipid free apoE3 incubated with stimulated normal fibroblasts generated pre-β-LpE3 with a particle size ranging from 9 to 15 nm (28). Interestingly, we found that pre-β-LpE3 contain 4 and 8 molecules of apoE3 per particle, whereas lipid-free apoE3 incubated with ABCA1 mutant (C1477R) remained in the monomeric form (data not shown). It is likely that the minimum functional unit of ABCA1 is a tetramer which lipidates 4 molecules of apoA-I at the same time, whereas the presence of 8 molecules of apoA-I per particles could be explained by the close proximity of two homo-tetrameric ABCA1 subunits. This is in agreement with our observation that a significant proportion of ABCA1 exists in living cells as an oligomer higher than tetramer (Fig. 1). Thus, the oligomeric ABCA1 complex generated multimeric nascent HDL particles regardless of the apolipoprotein acceptor.

We have assumed that the presence of LpA-I particles having either 4 or 8 molecules of apoA-I per particle is an accurate reflection of the presence of different LpA-I subpopulations generated by the oligomeric ABCA1 complex. It is possible, however, that the heterogeneity of LpA-I particles was in part due to the cross-linking procedure itself (i.e., inter-molecular cross-linking may occurred between separate LpA-I particles giving rise to apparent higher order oligomers of apoA-I which were not normally produced by ABCA1). Although this possibility cannot be totally excluded, experiments with different molar ratio of DSP to apoA-I and experiments involving a cross-linking at 37°C or room temperature rather than 4°C did not result in significant alteration of the number of apoA-I molecules per particle.

Although ABCA1 mutants Q597R and C1477R were found to oligomerize normally (data not shown) and localized to the plasma membrane, they showed total absence of binding to apoA-I (21,23). These results indicate that apoA-I lipidation defect observed in either Q597R or C1477R ABCA1 mutants is not caused by impaired oligomerization of ABCA1. Furthermore, C1477R, a naturally occurring mutant of ABCA1 in which cysteine 1477 within the second large extracellular loop is replaced with arginine (10), was found to dimerize normally. This suggests that cysteine 1477 is not essential for ABCA1 homodimerization. We are currently investigating the structural requirements for the ABCA1 transporter to form oligomeric complex.

It is well documented that phosphorylation of a number of cellular receptors, triggers their oligomerization, which modulates their function. Recent studies from our laboratory and others have shown that ABCA1 phosphorylation by the cAMP/PKA-dependent

pathway plays an important role in the apoA-I lipidation process (21,23,32,33). It is possible that apoA-I induces ABCA1 phosphorylation allowing ABCA1 oligomerization. Although apoA-I binds to both the dimeric and tetrameric ABCA1, the presence or absence of apoA-I molecules did not affect the oligomerization of ABCA1 in our cell culture model (Fig. 2B and Fig. 3A, respectively). More thorough investigations are required to establish definitively a possible role of apoA-I in the ABCA1 oligomerization process.

The molecular organization of apoA-I molecules within nascent LpA-I particles formed by the lipid translocase activity of the oligomeric ABCA1 complex has not yet been determined. However, because of the absence of cholesterol acyltransferase activity in the extracellular medium to convert unesterified cholesterol to cholesteryl ester, it is most likely that nascent LpA-I particles are discoidal. We have previously suggested that the α-electrophoretic mobility of LpA-I particles may be attributable to their high content in phosphatidylinositol (20,34). However, it is possible that the high number of apoA-I molecules per particle as documented in the present study may contribute to increase the net negative charge of LpA-I particles and consequently cause their α-electrophoretic mobility. Although the spatial organization of apoA-I molecules within nascent α-LpA-I particles is unknown, Segrest and colleagues (35) published a computer model referred to as the "double belt" model for reconstituted LpA-I particles containing two molecules of apoA-I. In this model, two ring-shaped molecules of apoA-I are stacked on top of each other forming a continuous amphipathic α helix that wraps around the perimeter of the phospholipid disc in an anti-parallel orientation resulting in the greatest potential for salt bridge connections between the two-molecules. It is likely that the conformation(s) of two apoA-I molecules assumed on 96 Å discs might not be the same as that found on nascent LpA-I containing 4 or 8 molecules of apoA-I. Of interest, the presence of heterogeneous subspecies of nascent α-LpA-I having both different size and number of apoA-I molecules support the idea that apoA-I conformations on discoidal particles is highly flexible (36). However, we wish to make clear that no attempt was made to use these models to give definitive interpretation concerning the organization of apoA-I molecules within nascent α-LpA-I. Our model presented in Fig. 6 is a simple illustration of apoA-I lipidation by the oligomeric ABCA1 complex. Detailed structural organization of apoA-I within these nascent particles requires more thorough investigations currently ongoing.

The results presented in this study provide a biochemical basis for a cellular apoA-I lipidation pathway that involves oligomeric ABCA1 complex in peripheral cells. This process plays *in vivo* a key functional role in the biogenesis of nascent HDL particles.

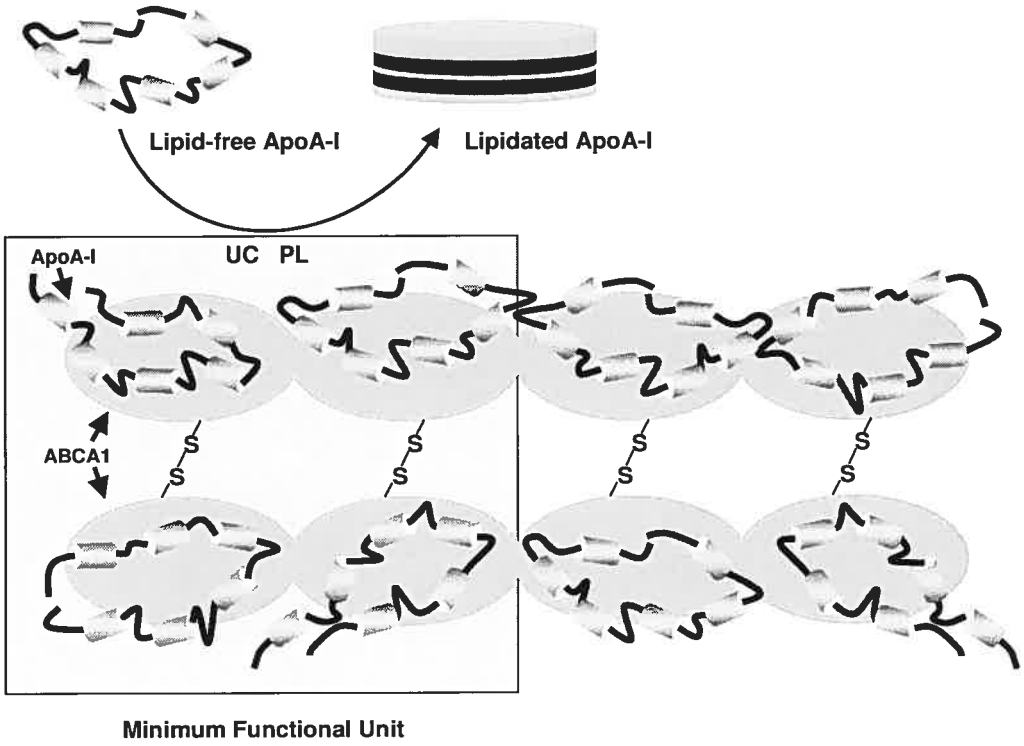


Figure 6. A proposed model of lipid-free apoA-I lipidation by the oligomeric ABCA1 complex in peripheral cells. Different types of molecular interactions exist between ABCA1 molecules. It is likely that the sulfhydryl groups (SH) between the ABCA1 subunits form disulfide bonds. There is no evidence of intermolecular disulfide bonds that link either a homo-dimeric or the homo-tetrameric ABCA1 subunits together. The homo-tetrameric complex designated as the minimum functional unit binds four molecules of apoA-I at the same time, followed by the transfer of phospholipids and cholesterol from the active site of the oligomeric ABCA1 complex allowing the assembly of 4 molecules of apoA-I on the same particle (Lipidated apoA-I). The transfer of lipids to apoA-I molecules may weakens the interaction of nascent LpA-I with the oligomeric ABCA1 complex and causes dissociation of the lipidated product. The organization of apoA-I molecules within nascent α-LpA-I is unknown. The present organization of apoA-I within LpA-I is a simple illustration of apoA-I lipidation by the oligomeric ABCA1 complex. PL, phospholipids; UC, unesterified cholesterol.

ACKNOWLEDGMENTS

The authors thank Karl H. Weisgraber for kindly providing apoE3. This work was supported by grants MOP 15042 from the Canadian Institutes of Health Research (CIHR), and the Heart and Stroke Foundation of Quebec. J. Genest holds the McGill University-Novartis Chair in Cardiology.

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Chapitre VI

Discussion

VI.1 Introduction

Il convient maintenant de faire un retour sur les aspects développés dans les trois chapitres précédents. Pour chacun seront respectivement discutés leur contribution à l'avancement des connaissances, une auto-critique sur le travail et ses limitations ainsi que des propositions de directions futures. Cette discussion permettra de mettre en lumière l'apport de ce travail à l'établissement de nouvelles avenues de recherche.

VI.2 Régulation transcriptionnelle

Contribution

Comparativement à la découverte et à la caractérisation par d'autres groupes des éléments de régulation DR4 et E-box dans le promoteur, l'article #1 (chapitre 3) pourrait sembler n'apporter qu'une modeste contribution. Toutefois, il a permis d'établir :

1) le mécanisme de régulation de l'ABCA1 par les stérols dans les fibroblastes;
2) que l'AMPc ne s'impose plus comme stimulateur universel de la transcription d'ABCA1;
3) un modèle simple d'étude de l'interaction apoA-I/ABCA1 à l'aide du système inductible naturel des fibroblastes (stimulation par 22OH/9CRA).

Ceci était un pré-requis essentiel à la poursuite de nos études.

Auto-critique

La compréhension du mécanisme de régulation génique est pré-requis à l'établissement d'un modèle permettant d'étudier la fonction de l'ABCA1. Toutefois, plusieurs points restent inexpliqués dans cette étude. Par exemple, bien qu'un inhibiteur de la voie LXR/RXR (GGPP) suppose la conversion du cholestérol en hydroxystérol, une régulation directe du cholestérol via la SREBP ne peut être exclue. Ce facteur de transcription inhibé en présence de cholestérol et activé en cas contraire pourrait agir comme répresseur de

la transcription d'ABCA1 en absence de cholestérol. La surcharge en cholesterol lèverait donc l'inhibition. Enfin, le mécanisme de régulation différentielle entre les tissus humains et murins par l'AMPc reste indéterminé.

Expériences supplémentaires

Afin de déterminer un rôle possible des éléments SRE dans la régulation par les stérols, deux approches peuvent être employées. D'abord, la suppression successive des différents éléments du promoteur d'ABCA1 cloné devant un gène rapporteur (luciférase) permettrait de mieux comprendre la régulation d'ABCA1 par les stérols. Il est difficile de croire que les trois ou quatre éléments SRE présents dans le promoteur ne contribuent en rien à la régulation du gène. Il est aussi possible d'utiliser une co-transfection du SREBP activé avec la construction promoteur/gène rapporteur afin de déterminer la contribution de ce facteur à l'inhibition de la transcription. Enfin, l'utilisation de la technique de précipitation de la chromatine réalisée sur des fibroblastes à l'aide d'un anticorps anti-SREBP permettrait d'identifier les séquences régulatrices nécessaires à la liaison du facteur de transcription.

Afin de déterminer le mécanisme par lequel l'AMPc régule l'expression d'ABCA1, une étude différentielle de liaison des facteurs de transcription au promoteur pourrait être faite (« footprinting »). Il serait ainsi possible de comparer les propriétés de liaison des facteurs de transcription provenant d'extraits nucléaires de macrophages et de fibroblastes. Par ailleurs, les différents mutants de délétion de la construction promoteur/gène rapporteur mentionnée précédemment pourraient être transfectés dans différents types cellulaires (fibroblastes vs macrophages). Ceci permettrait de complémenter l'étude de régulation différentielle en identifiant les éléments requis à la régulation par l'AMPc dans un tissus plutôt que dans un autre.

Perspectives

Dans la course à la mise sur pieds de médicaments augmentant les HDL, l'ABCA1 représente une cible de choix. La compréhension de sa régulation pourrait avoir un impact majeur sur le développement de médicaments activant sa transcription d'une façon tissu-spécifique. Le LG268, un composé réxinoïde activant spécifiquement le RXR, a démontré dans les souris une excellente capacité à réduire l'absorption intestinale du cholestérol et à activer la transcription d'ABCA1. Aussi, ce composé peut être utilisé en

combinaison avec des composés non-stéroidiens comme le T0901317 qui activent spécifiquement le LXR et, conséquemment, la transcription d'ABCA1 [159]. Par ailleurs, Oliver et coll. ont élaboré un agoniste de PPARδ, le GW401516, capable d'augmenter la transcription de l'ABCA1 et l'efflux de cholestérol médié par l'apoA-I. Les trois sous-types de facteurs nucléaires PPAR (α, δ, γ), sont exprimés dans des tissus différents. Alors que le sous-type α est majoritairement exprimé dans le foie, le sous-type γ est plutôt concentré dans le tissu adipeux, et le sous-type δ est dispersé dans les autres tissus. Avec les fibrates comme activateurs sélectif de PPARα et les composés glitazones activant le PPARγ [160], le GW401516 représente un outil ouvrant une porte supplémentaire dans l'élaboration d'une stratégie de traitement ciblé.

VI.3 Caractérisation de l'interaction apoA-I/ABCA1

Contribution

L'interaction entre l'apoA-I et l'ABCA1 est sujet à de nombreux débats. La littérature propose deux modèles d'interaction entre les deux molécules. Notre explication de l'interaction propose un contact direct protéine/protéine de type récepteur/ligand. Ceci est basé sur l'observation que ni la phosphatidylcholine ni la sphingomyéline ne sont requises à l'association de l'apoA-I avec l'ABCA1. Cette observation, combinée à celle de Smith et coll. qui montrent que la phosphatidylsérine n'est pas impliquée non plus dans la liaison, constitue une ***observation importante*** pour la caractérisation de cette interaction. Par ailleurs, il a été supposé, sans jamais être démontré, que la lipidation de l'apoA-I diminue son affinité pour l'ABCA1. À l'aide d'un essai de compétition de liaison, nous avons été ***les premiers à le démontrer***. Finalement, malgré ce que les articles de revues les plus récents supposent [161], le concept que les particules pré-β soient le résultat de la lipidation de l'apoA-I par l'ABCA1 n'est, chez les fibroblastes, pas exact. ***Nous sommes les premiers*** à avoir caractérisé les produits de dissociation du complexe apoA-I/ABCA1 : il s'agit de particules ayant une migration alpha. Ceci constitue une ***révélation majeure qui bouleverse le modèle établi***. L'originalité de notre travail réside dans le fait que notre caractérisation provient spécifiquement de l'interaction apoA-I/ABCA1 et permet d'expliquer plusieurs observations faites avant l'identification du transporteur.

Discussion supplémentaire

Les bouleversements majeurs qu'amènent les résultats présentés dans cet article proviennent de la caractérisation des particules résultantes de la lipidation de l'apoA-I par l'ABCA1. Nous avons montré que l'apoA-I dissociée de l'ABCA1 est phospholipidée et migre en position alpha. Les études précédentes réalisées par Castro et Fielding en 1987 avaient proposé que les particules pré-β étaient les premières à acquérir du ^{3}H-cholestérol et ce, après seulement une minute d'incubation du sérum avec les cellules radiomarquées. Après la troisième minute, la radioactivité était détectée dans les particules alpha. Sur la base de ces observations sommaires, un modèle complet de lipidation de l'apoA-I a été établi qui fit dogme de foi jusqu'à aujourd'hui. Ce modèle proposait que l'apoA-I délipidée pouvait acquérir du cholestérol et des phospholipides pour former une particule pré-β discoidale. Ensuite, sous l'action de la LCAT, le cholestérol serait estérifié et, se dirigeant vers l'intérieur de la particule, la rendrait plus sphérique et avec une migration en position alpha [53].

En 1997 cependant, toujours avant l'identification de l'ABCA1, Asztalos et coll. ont fait une étude qui supporte nos résultats. En effet, ils ont observé que l'apoA-I délipidée ayant une migration en pré-β se transformait, après une incubation avec des fibroblastes radiomarqués, en des particules contenant du cholestérol, des phospholipides, et migrant en pré-α [162].

Aussi, supportant notre concept, von Eckardstein et coll. ont comparé par gel bi-dimensionnel le profil des particules LpA-I (particules contenant l'apoA-I) provenant du plasma d'un sujet contrôle, d'un sujet Tangier, d'un sujet déficient en apoA-I et d'un sujet déficient en LCAT. Il est intéressant de constater que le patient Tangier présente bel et bien des particules pré-β, mais pas de particules alpha. Si l'ABCA1 était responsable de la formation des particules pré-β, elles ne devraient pas exister chez ce sujet, contrairement à ce qui est observé. De plus, chez le patient avec une déficience en LCAT se forment (modestement) des particules alpha immatures, suggérant que la génération *in vivo* de particules α-LpA-I ne requiert pas la LCAT. En fait, l'estérification du cholestérol n'est pas en cause pour expliquer la migration en alpha. Si l'on en croit les études de Davidson et coll en 1994 [163], l'addition de lipides neutres sur l'apoA-I n'affecte pas son potentiel de surface. De plus, selon Zhao et coll. [164], ce ne serait ni la quantité de

phospholipides, ni la quantité de cholestérol, ni même celle de monomères d'apoA-I qui favoriserait la migration en position alpha; ce serait plutôt la quantité de phosphatidylinositol, le seul phospholipide possédant une charge nette négative.

Récemment, Zannis et coll. ont étudié le plasma de souris infectées avec un adénovirus encodant une apoA-I dont les hélices 9 et 10 étaient supprimées. Par gel bi-dimensionnel, il a été observé que cet apoA-I muté formait bel et bien des pré-β, et non des alpha. Il s'agit d'un résultat quasi identique à celui obtenu de patients Tangier. Ceci signifie qu'une mutation dans le récepteur (ABCA1), ou la suppression des hélices 9 et 10 du ligand n'empêche pas la formation des pré-β, mais seulement la formation des alpha [9].

Finalement, nous avons montré (Krimbou et coll.) que l'interaction apoA-I/ABCA1 est très spécifique. En effet, le résultat d'une interaction entre l'apoE et l'ABCA1 est une particule demeurant en position pré-β, et ce malgré qu'elle soit associée à du ^{14}C-cholestérol et des ^{32}P-phospholipides [151].

Auto-critique

Au moins trois questions sont laissées sans réponses dans l'article #2 présenté au chapitre IV. La première est due à l'absence d'une caractérisation détaillée des particules alpha résultantes de l'interaction apoA-I/ABCA1. Des particules de tailles différentes sont observées en position alpha. Cependant, rien n'indique s'il s'agit de particules composées de deux, trois ou quatre molécules d'apoA-I (l'article #3 présenté au chapitre V répond toutefois à cette question), si elles sont sphériques ou discoïdales, si elles représentent un bon substrat pour la LCAT, etc.

La deuxième est l'origine *in vivo* des pré-β. D'où viennent-elles? Tout porte à croire qu'elles sont formées par un mécanisme ABCA1-indépendant. Alors, comment deviennent-elles lipidées? Cela suppose un mécanisme de microsolubilisation, mais cela n'est pas discuté en détail.

La dernière est la faible concentration plasmatique de l'apoA-I délipidée *in vivo*. Nos résultats suggèrent que l'apoA-I délipidée est le meilleur ligand pour l'ABCA1. Viennent ensuite les pré-β discoïdales, puis les particules reconstituées et les HDL. Toutefois, *in vivo*, la demi-vie d'une molécule d'apoA-I non lipidée est négligeable. La riche présence

d'hélices alpha amphipatiques rend cette molécule avide de lipides pour probablement former des pré-β. Alors, quel est *in vivo* le ligand d'ABCA1? L'affinité des pré-β pour l'ABCA1 est bien meilleure que celle des HDL, et elles sont présentes même chez les patients Tangier (voir discussion ci-haut). *Il se pourrait* qu'*in vivo* les pré-β soient le ligand majoritaire de l'ABCA1.

Expériences supplémentaires

Plusieurs expériences de caractérisation des particules dissociées sont présentement en cours dans le laboratoire. Par exemple, la capacité de ces particules à former des HDL matures via la LCAT est sous investigation. Aussi, afin d'établir un modèle cohérent de genèse des différentes particules alpha retrouvées, il faudrait en déterminer la composition en phospholipides et en cholestérol. Finalement, il serait intéressant d'identifier les résidus de l'apoA-I responsables du contact protéine/protéine avec l'ABCA1. Pour ce faire, deux stratégies peuvent être employées. La première est d'utiliser des mutants de délétion de l'apoA-I dans des essais de compétition de liaison. La deuxième pourrait s'apparenter à celle utilisée par Davidson et coll. pour identifier, dans le modèle en épingle à cheveux (figure I.8 C), la position de chaque hélice par rapport aux autres. Dans leur approche, ils ont effectué un pontage moléculaire (cross-linking) suivi d'une digestion à la trypsine. Les peptides pontés résultants étaient ensuite identifiés par spectroscopie de masse [165].

VI.4 Oligomérisation d'ABCA1

Contribution

Jusqu'à maintenant, l'ABCA1 avait toujours été vue comme fonctionnant sous forme de monomère. Au niveau bactérien, un transporteur ABC fonctionnel requiert l'assemblage de sous-unités membranaires et de domaines de liaison aux nucléotides. Chez les organismes supérieurs, les ABC sont pré-assemblés en une seule molécule, sauf exceptions (les hémitransporteurs). Encore une fois, ces protéines doivent homo/hétéro-dimériser afin de pleinement s'acquitter de leur tâche. Mais rien ne laissait présager que le transporteur complet ABCA1 pourrait former un niveau supérieur d'organisation. Ainsi, nos travaux ont ***complètement changé le concept*** de fonctionnement de l'ABCA1. La protéine sera dorénavant considérée comme faisant partie d'un complexe fonctionnel

composé de plusieurs monomères. De plus, ce n'est que sous sa forme oligomérique que l'ABCA1 peut lier l'apoA-I. Finalement, ***nous avons démontré*** que cette forme d'organisation supérieure se traduit en un aspect dynamique par la formation de particules alpha composées d'apoA-I dimérique, trimérique, tétramérique et peut-être même octamérique!

Auto-critique

Notre étude démontre bien que l'ABCA1 peut s'associer en homodimères par la formation de ponts disulfures intermoléculaires. Toutefois, elle n'apporte qu'une preuve indirecte qu'il s'agit bien d'un homodimère : c'est l'absence d'autre bande majeure marquée au ^{35}S qui suppose l'homodimérisation. De plus, à part la liaison du ligand, rien n'indique que l'oligomérisation soit requise pour la fonction de l'ABCA1 : lipider l'apoA-I. Finalement la forme que prennent les molécules issues du contact apoA-I/ABCA1 n'a pas été déterminée et il est difficile d'imaginer qu'une particule HDL contienne huit molécules d'apoA-I, tel que suggéré par la figure 5 de l'article #3 (chapitre V).

Expériences supplémentaires

Deux autres expériences pourraient confirmer que l'oligomère est composé de monomères d'ABCA1. La première serait de co-transfecter des cellules COS (par exemple) avec deux constructions d'ABCA1, l'une comportant un épitope myc (par exemple), l'autre épitope hémagglutinine (HA). Une immunoprécipitation en utilisant un anticorps contre un épitope, suivie d'une révélation immunologique en utilisant l'autre épitope constituerait une suggestion positive d'une homodimérisation. Aussi, d'une façon similaire, une construction ABCA1-luciférase ainsi qu'une construction ABCA1-YFP (« yellow fluorescence protein ») pourraient par « bioluminescence resonance energy transfer » (BRET) démontrer que ces deux constructions interagissent, et donc qu'il y a homodimérisation. Finalement, il serait possible de muter successivement les cystéines des domaines extracellulaires de l'ABCA1 jusqu'à la disparition de la dimérisation. Cette construction pourrait alors être aussi utilisée pour tester la capacité de la protéine mutée à générer des particules alpha et à effluer les lipides.

VI.6 Proposition d'un modèle intégré

Afin de résumer les choses, voici un modèle qui tente d'intégrer les notions présentées. Plusieurs étapes sont spéculatives, d'autres s'appuient sur les données de la littérature et celles présentées ici. Un résumé du modèle proposé est présenté à la figure VI.1.

Nos résultats présentés au chapitre V (article 3) suggèrent que dès sa traduction dans le réticulum endoplasmique, l'ABCA1 formerait probablement des oligomères. De cet endroit le transporteur migrerait vers la membrane plasmique où il lierait l'apoA-I pauvrement lipidé, ou même des pré-β, conformément aux résultats obtenus dans le chapitre IV (article 2). Cet association activerait la phosphorylation de la séquence PEST, évitant la dégradation du transporteur par la calpaïne, tel que montré par Wang et coll. La protéine kinase A (PKA) phosphorylerait alors le transporteur [135], favorisant son interaction avec des protéines adaptatrices inconnues. Cette interaction enclencherait l'internalisation du complexe. Une fois endocytée, on peut imaginer que l'ABCA1 puisse transporter activement des phospholipides sur l'apoA-I pauvrement lipidée. Cette opération pourrait favoriser l'association de plusieurs molécules d'apoA-I (voir chapitre V) en une seule particule qui se dissocierait, conséquence d'une diminution d'affinité suite à la lipidation (voir chapitre IV). Il est possible qu'alors, un apport des vésicules endocytiques de radeaux lipidiques (« rafts ») permette le transfert du cholestérol vers les particules phospholipidées, une étape qui serait indépendante de l'ABCA1. La vésicule pourrait fusionner alors à la membrane où une particule α-Lp4A-I, i.e. une particule HDL immature, serait relâchée, tel que démontré dans le chapitre V. Des phosphatases pourraient alors déphosphoryler l'ABCA1 qui serait ainsi prête pour un nouveau cycle de lipidation. La particule α-Lp4A-I, au contact de la LCAT plasmatique, pourrait former des HDL_3 matures.

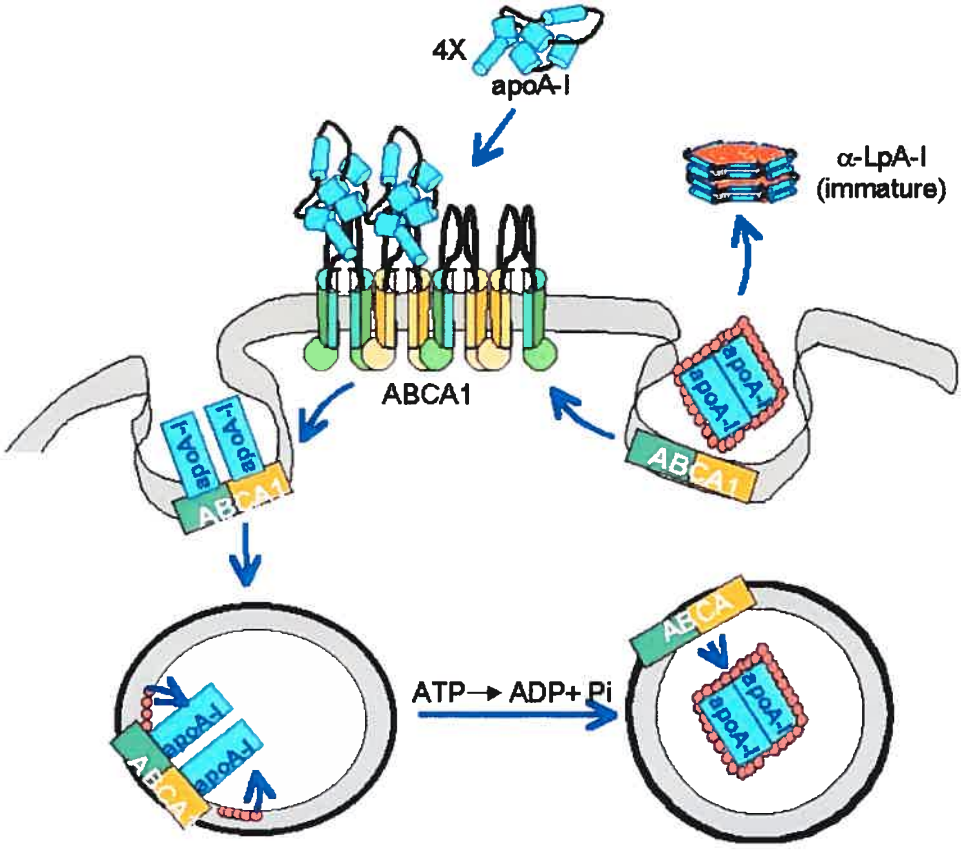

Figure VI.1 : **Proposition d'un modèle intégré du fonctionnement de l'ABCA1**. Voir le texte pour l'explication.

Chapitre VII

Conclusion

Chaque morceau ajouté au casse-tête permet de discerner de mieux en mieux l'image globale du mécanisme de fonctionnement de l'ABCA1. Cette thèse représente, certes, beaucoup de persévérance et de collaborations, mais ce travail rapporte énormément de fruits. D'abord, chaque partie du travail présente des éléments nouveaux sur lesquels bâtir de nouvelles expériences. Ensuite, un modèle général récapitulatif propose un concept sur lequel peuvent s'élaborer de nouvelle théories et de nouveaux projets. Plusieurs aspect cependant, se doivent d'être validés. Finalement, pour replacer cela dans un contexte métabolique, la contribution à la compréhension du fonctionnement de l'efflux de cholestérol et à la génération des particules HDL a un potentiel majeur quant aux perspectives de nouvelles thérapies.

Pour ne pas être en reste, et pour terminer ceci sur un note humoristique, Beulens et coll. ont fait une étude d'efflux de cholestérol en utilisant le sérum d'hommes adultes ayant consommé ou non une quantité modérée (4 verres/jour) d'alcool pendant 17 jours. Leur étude « en double aveugle » a révélé que le sérum des consommateurs de whisky augmentait de 17.5% l'efflux de cholestérol ABCA1-dépendant provenant de cellules J774. Cet efflux serait corrélé avec une augmentation de l'apoA-I sérique [166]. Ces résultats intéressants, combinés aux importantes masses d'argent investies à la recherche sur le transport à rebours du cholestérol indiquent que *l'avenir semble tout à fait bienheureux à celui qui cherche à augmenter les niveaux de HDL-cholestérol...*

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Molecular Genetics and Metabolism 78 (2003) 265–274
Molecular Genetics and Metabolism
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Expression, regulation, and activity of ABCA1 in human cell lines

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Received 29 August 2002; received in revised form 10 January 2003; accepted 10 January 2003

Abstract

Mutations in the ATP-binding cassette transporter A1 (ABCA1) gene cause familial high-density lipoprotein deficiency and Tangier disease. ABCA1 plays a crucial role in active apolipoprotein A-I (apoA-I) lipidation, a key step in reverse cholesterol transport. We compared ABCA1 transcriptional regulation and cholesterol efflux in human skin fibroblasts, monocyte-derived macrophages and hepatocytes (HepG2). 8-Br-cAMP did not increase ABCA1 transcription in these tissues compared to mouse macrophages. We found that ABCA1 is differentially regulated among tissues. While transcription in HepG2 appears to be constitutive, sterols stimulate ABCA1 transcription in fibroblasts and monocyte-derived macrophages. ApoA-I promoted cholesterol efflux in fibroblasts, macrophages, and HepG2. Cholesterol homeostasis in fibroblasts is tightly regulated, and ABCA1 mRNA closely follows the cellular mass of free cholesterol (dose- and time-dependent manner). To further determine the mechanism used by fibroblasts to maintain sterol balance, we used a competitive inhibition approach with geranylgeranyl pyrophosphate (GGPP) to block the LXR induction pathway. GGPP blocked basal, 22-(*R*)-hydroxycholesterol- and cholesterol-induced ABCA1 expression. Taken together, these results demonstrate that: (1) ABCA1 expression varies among tissues, and (2) cholesterol conversion to hydroxycholesterol is an important mechanism for the maintenance of cholesterol homeostasis in fibroblasts.

Keywords: ABCA1; Cholesterol; Hydroxysterol; Gene regulation; Fibroblast

The ABCA1[1] gene codes for the ATP-binding cassette transporter A1 required for the efflux of phospholipids and cholesterol from cells. Heterozygous patients for mutations at the ABCA1 gene locus cause familial HDL deficiency (FHD) whereas the homozygous or compound heterozygous forms cause Tangier disease (TD) [1–5]. The ABCA1 protein is thought to promote active transport of phospholipids and cholesterol to the plasma membrane where they become available for efflux onto acceptor particles [6]. The main physiological acceptors for efflux are lipid poor apoA-I particles, the precursors of HDL. Absence of functional ABCA1 leads to a marked reduction in apoA-I-mediated cellular cholesterol efflux and the lack of formation of mature, spherical HDL [7]. Immature particles are then rapidly catabolized, most likely in the kidney or liver, causing low HDL levels [6]. Both the homozygous and heterozygous forms are associated with an increased risk of coronary artery disease [8,9].

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[1] *Abbreviations used:* ABCA1, ATP-binding cassette transporter A1; BSA, bovine serum albumine; BAC, bacterial artificial chromosome; TD, Tangier disease; FHD, familial HDL deficiency; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FBS, fetal bovine serum; LPDS, lipoprotein deficient serum; cAMP, cyclic adenosine monophosphate; LXR, liver X receptor; RXR, retinoic X receptor; 9CRA, 9-*cis*-retinoic acid; 22OH, 22-(*R*)-hydroxycholesterol; HSF, human skin fibroblasts; FC, free cholesterol; CE, cholesteryl ester; ApoA-I, apolipoprotein A1; DR, direct repeat; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; PMSF, phenylmethylsulfonide fluoride; PVDF, polyvinylidene fluoride; SDS–PAGGE, sodium dodecylsulfate–polyacrylamide gradient gel electrophoresis.

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doi:10.1016/S1096-7192(03)00020-9

The ABCA1 transporter is predicted to have 12 transmembrane domains and to be synthesized with a signal peptide [10,11]. As in other ABC transporters, two intracellular segments of the protein contain nucleotide-binding domains (NBDs) that allow the protein to bind and slowly hydrolyse ATP [12]. The extracellular portion is glycosylated [11] and the transporter appears to shuttle between late endosomal compartments and the plasma membrane [13], where ABCA1 is thought to directly [14–16] or indirectly [17] interact with apoA-I. Studies on ABCA1 gene regulation performed in mouse or human macrophages have revealed that cAMP analogs [14,15,18], modified LDL [19], or hydroxycholesterol [20–22] induce ABCA1 transcription. The regulation by hydroxysterols is explained by the presence of functional DR4 elements in the promoter and in intron 1 [23] of the ABCA1 gene. These elements allow the binding of a heterodimer composed of the liver-X-receptor (LXR) and the retinoid-X-receptor (RXR) and enhance the transcription of the gene. Although the gene is expressed in most cell types, many studies on the regulation of its expression have concentrated on mouse macrophages. This is probably due to the observation that cholesterol-laden macrophages were found to accumulate in lymphoid tissues from Tangier disease patients [6]. However, a recent report suggests that macrophage-specific ABCA1 does not contribute significantly to plasma HDL levels [24]. This finding suggests that ABCA1-mediated lipid efflux from other tissues contributes to HDL-cholesterol levels.

For many years, human skin fibroblasts (HSF) have been used as a model to study cellular cholesterol homeostasis and, more recently, cellular cholesterol efflux. Cholesterol homeostasis is maintained in fibroblasts by four mechanisms: (1) cholesterol influx by the LDL-receptor pathway; (2) de novo synthesis by the HMG-CoA reductase pathway; (3) equilibrium between esterified and free cholesterol by the acyl-CoA cholesterol acetyltransferase (ACAT); and (4) ABCA1-mediated cholesterol efflux [25]. Unlike fibroblasts, macrophages can become foam cells. This is partly due to the expression of scavenger receptors for modified lipoproteins that are not transcriptionally regulated by sterols [26]. This suggests that cellular lipid metabolism is regulated differently in that cell type. Indeed, fibroblasts do not produce large amounts of oxysterols, as is the case in macrophages, hepatocytes, and adrenal cells. Moreover, oxysterols are toxic to some fibroblast and endothelial cell lines [27,28].

Based on these considerations, we studied the ex vivo regulation of endogenous ABCA1 in human cell lines and focused on human skin fibroblasts. We found that the conversion of cholesterol to hydroxycholesterol is an important mechanism to regulate sterol homeostasis in that cell type.

Methods

Materials

All reagents for cell culture were from Gibco (Invitrogen), and all the others were from Sigma. Avasimibe (CI-1011) was a generous gift of Pfizer. Hydroxysterols and cholesterol were dissolved in ethanol at a concentration of 10 mg/ml. 8-Br-cAMP was dissolved in water at a concentration of 0.23 M. HSP70 antibody was from Transduction Laboratory, Lexington, KY.

Cell culture

Primary cultures of HSF obtained from punch biopsy were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, non-essential amino acids, and 10% fetal bovine serum (FBS). J774 cells were grown in RPMI1640 medium containing glutamine to which were added 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS. HepG2 cells (ATCC) were grown in DMEM-F-12, pH 7.15, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% fetal bovine serum (FBS). Trypsin (0.25%), 0.03% EDTA solution was used to separate the cells and 500,000 cells were seeded in 60 mm plates coated with 6 μg/cm^2 rat tail collagen type I, as described by Dixon and Ginsberg [29]. All inductions were performed in medium + BSA 2 mg/ml without serum for 24 h.

Macrophage isolation

Monocyte-derived macrophages from a control subject were isolated on Ficoll–Paque gradient (Amersham/Pharmacia), following manufacturer's protocol. Briefly, 136 ml of blood was mixed with an equal volume of a balanced salt solution (NaCl 126 mM, 0.01% D-glucose, $CaCl_2$ 5 μM, $MgCl_2$ 98 μM, KCl 0.54 mM, Tris 14.5 mM, pH 7.6) and layered on top of Ficoll–Paque gradient. Following centrifugation (400*g* for 30 min), the white layer corresponding to leukocytes was extracted, washed three times in balanced salt solution, once in RPMI medium, and the cells were seeded in 60 mm petri dishes (15 million cells/dish) in RPMI medium containing 10% autologous serum. Monocytes were allowed to attach to the bottom of the dish for 16 h. At that time, a wash with culture medium removed non-adherent lymphocytes. Monocytes were treated for 7 days with macrophage-colony stimulating factor (M-CSF) (10 ng/ml) (Peprotech, CA) and controlled for differentiation into macrophages by testing for the CD68 antigen expression by Western blot (antibody from Dako, Mississauga, Ont., Canada).

Serum and lipoprotein preparation

LPDS was prepared by ultracentrifugation of FBS using previously described methods [30]. Low-density lipoprotein (LDL) and high-density lipoprotein subfraction 3 (HDL_3) were obtained by potassium bromide density gradient ultracentrifugation by a previously described protocol [30] on human serum collected from healthy donors.

Cholesterol efflux

Performed as described [1]. Briefly, half-confluent cells were labeled with [^{3}H]cholesterol (0.2 μCi/ml) and grown until confluence. Cells were then washed five times with PBS/BSA and loaded with free cholesterol (20 μg/ml) for 24 h in DMEM/BSA 2 mg/ml. Cells were washed two times with PBS/BSA and allowed to equilibrate for 24 h in DMEM/BSA 1 mg/ml. A 24 h efflux was performed by changing medium to DMEM/BSA 1 mg/ml containing (or not) 10 μg/ml delipidated apoA-I. The efflux medium was recovered, cells were lysed in NaOH 0.1 N, and aliquots from the medium and cells were counted for β-scintillation. The percentage of efflux was determined as the percent counts in medium over counts in medium + cells.

Cellular cholesterol mass measurements

Performed as described [31], with minor modifications. Briefly, cells were washed two times in ice-cold PBS/BSA 1 mg/ml, twice in ice-cold PBS, and lipids were extracted for 30 min at room temperature in 5 ml of hexane:isopropanol (3:2 v/v). The extraction was repeated in 3 ml and the extraction media were combined and evaporated in 12 × 75 mm borosilicate glass tubes under nitrogen. The extraction medium contained 25 μg of stigmasterol and 17.5 μg of stigmasteryl oleate to serve as internal standards for the gas chromatography step. Lipids were resuspended in chloroform and separated by thin-layer chromatography (Analtech, Silica Gel G) in an elution system constituted of heptane:ethyl ether:methanol:acetic acid (80:30:3:1.5). Spots corresponding to sterol and sterol esters were scraped and treated separately. Sterols were extracted from the powder by Folch (chloroform:methanol, 2:1) extraction. Steryl esters were hydrolysed by treatment in 0.5 ml of KOH 0.5 M in methanol for 30 min at 80 °C. The released sterols were recovered from the organic phase of an extraction in 1 ml hexane + 0.5 ml water. Both free sterols and steryl-ester-derived sterols were resuspended in chloroform and derivatized for 15 min at 80 °C, prior to loading on a gas chromatography column (Hewlett Packard). Cholesteryl mass for each spot was corrected by dividing by protein mass for each sample. All experiments were performed in triplicate.

Probes and Northern blots

A 517 bp probe for human ABCA1 and for mouse ABCA1 were prepared by reverse transcription performed on total RNA obtained from human skin fibroblasts and from J774 mouse macrophages. This was followed by a PCR step using the forward primer 5′-CCT TGG GTT CAG GGG ATT AT-3′ and the reverse primer 5′-AGG ATT GGC TTC TTC AGG ATG TCC-3′. The amplified fragment were subcloned into pGEM-T (Promega) and used to transform JM109 cells and sequenced to ensure the proper identity. Probes were prepared by digestion with *Sal*I and *Sac*II and the insert was excised out of the agarose gel and ^{32}P-labelled using the Amersham/Pharmacia Oligolabeling kit and used as a probe at a specific activity of 10^6 cpm/ml in Northern blots. A glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe was obtained using a similar strategy. Ten to fifteen micrograms/lane of total RNA extracted from cells using the RNEasy kit (Quiagen) were loaded on a 1% formaldehyde–agarose gel. RNA was transferred to a Hybond N+ (Amersham) membrane and probed. Bands were quantified on a Storm phosphorimager (Molecular Dynamics). To correct for loading, the signal of the bands was divided by the signal for the 18S ribosomal subunit, and the control condition was adjusted to 100%.

mRNA half-life

Ninety percent confluent HSF were grown for 24 h in DMEM containing 5% LPDS. The medium was changed to DMEM/BSA 2 mg/ml containing 20 μg/ml free cholesterol or vehicle alone (ethanol) for 24 h. The medium was then changed to DMEM containing the transcription inihibitor DRB (5,6-dichlorobenzimidazole riboside) (Sigma) [32] at a concentration of 10 μg/ml and incubated for the indicated times. The medium was removed and RNA extraction was performed using the RNEasy kit (Quiagen), prior to Northern blotting on 15 μg of total RNA. Bands were quantified on a Storm phosphorimager (Molecular Dynamics) and expressed as a ratio of the signal divided by the 18S ribosomal subunit, with the control condition adjusted to 100%. The interpolation and calculations of half-life values were performed using the GraphPad Prism 3.02 software (GraphPad Softwares Inc.)

ABCA1 antibody generation

A peptide encoding the amino acids 867-GEESDEKSHPGSNQKRISE-885 of the human ATP-binding cassette-1 derived from the published sequence (Accession No. O95477) was synthesized at the McGill University Sheldon Biotechnology Center (Montreal, PQ, Canada), according to the multiple antigenic

peptide (MAP) method of Tam [33]. New Zealand White rabbits were immunized with the peptide. The antibody was purified on a protein A–Sepharose coupled bead (Amersham/Pharmacia Biotech) column and eluted with glycine 0.1 M, pH 2.5. Fractions containing the antibody were pooled, dialyzed, and concentrated by centrifugation in a Centriplus 10 column (Amicon). The concentration of antibody was adjusted to 2.5 mg/ml in a mixture containing glycerol 50%, NaCl 150 mM, and BSA 1 mg/ml and stored at −20°C until further use.

Immunoblotting

The cells were cholesterol loaded in DMEM/BSA 2 mg/ml supplemented with 20 μg/ml of free cholesterol for the indicated times. The cells were washed twice in ice-cold PBS/BSA, twice in ice-cold PBS, and scraped in lysis buffer (20 mM Tris–HCl, 0.32 M sucrose, pH 7.4, 50 mM 2-mercaptoethanol, 0.2 mM PMSF, 20 μg/ml leupeptin, and 25 μg/ml aprotinin) and homogenized with a 2 ml tight-fitting dounce homogenizer. The homogenate was cleared of cell debris by gentle centrifugation (1000*g* for 10 min at 4 °C). The post-nuclear supernatant (PNS) was removed, stored on ice, and an aliquot was used for protein determination with Bradford reagent (Bio-Rad) according to manufacturer's instructions. Ten micrograms of PNS proteins were migrated on a 4–12.5% SDS–PAGGE and transferred to a PVDF-ImmobilonP membrane (Millipore). The membrane was blocked and incubated with our purified anti-ABCA1 antibody diluted 1:1000 in Tris-buffered saline containing Tween 0.5% (TBS-T) + 1% dehydrated for 90 min. The membrane was incubated for 90 min in TBS-T + 1% dried milk + 1:12,500 horseradish peroxidase-coupled rabbit secondary antibody (Pharmacia). Immunoreactive bands were revealed by chemiluminescence with the "Supersignal" reagent obtained from Pierce. The membrane was exposed for 1 min to a Kodak Xomat film.

Results

ABCA1 is differentially regulated in tissues

Recent studies have shown that macrophage ABCA1 is regulated at the transcriptional level by many agents such as AcLDL [19], cAMP analogs [14,15,18] and by the LXR/RXR agonists hydroxysterols and 9-*cis*-retinoic acid [20–22]. To determine whether ABCA1 is regulated in similar or different manners in all tissues, the effect of cholesterol (10 μg/ml), 8-Br-cAMP (0.3 mM), and 22-(*R*)-hydroxycholesterol (2.5 μg/ml) plus 9-*cis*-retinoic acid (10 μM) for 24 h was tested by Northern blot analysis in HSF, human monocyte-derived macrophages, and human hepatocytes (HepG2). As shown in Fig. 1, cholesterol and 22OH together with 9CRA were strong modulators of ABCA1 mRNA in fibroblasts, but not necessarily in other cell types. In contrast to HSF, cholesterol alone was not a potent modulator of ABCA1 mRNA in macrophages or HepG2. Also, 8-Br-cAMP was a strong modulator of ABCA1 mRNA expression in J774 murine macrophages whereas it had little or no effect on ABCA1 mRNA levels in human tissues, strongly arguing for a differential regulation between cell types and species. Fig. 2 also shows that ABCA1 function also correlates with mRNA levels in most tissues, with the conclusion that whenever ABCA1 is present and modulated, an apoA-I-specific cholesterol efflux can occur.

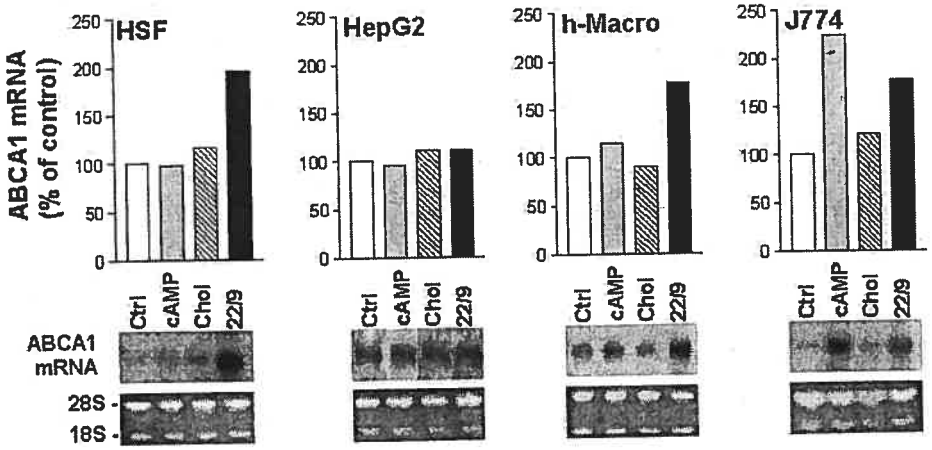

Fig. 1. Differential regulation of ABCA1 in human and mouse tissues. Human skin fibroblasts (HSF), HepG2 cells (HepG2), human monocyte-derived macrophages (h-Macro) and mouse macrophages J774 cells (J774) were incubated for 24 h in medium/BSA 2 mg/ml containing either vehicle (ctrl), cholesterol 10 μg/ml (chol), 8-bromo-cyclic adenosine monophosphate (cAMP) 0.3 mM, 22-(*R*)-hydroxycholesterol 2.5 μg/ml + 9-*cis*-retinoic acid 10 μM (22/9). Total RNA was extracted and a Northern blot was performed using an anti-human or anti-mouse ABCA1 probe. Blots were quantified and results are expressed as a percentage of the control condition for each cell line. 28S and 18S ribosomal subunits were used to control for loading. The blot shown is representative of two different experiments.

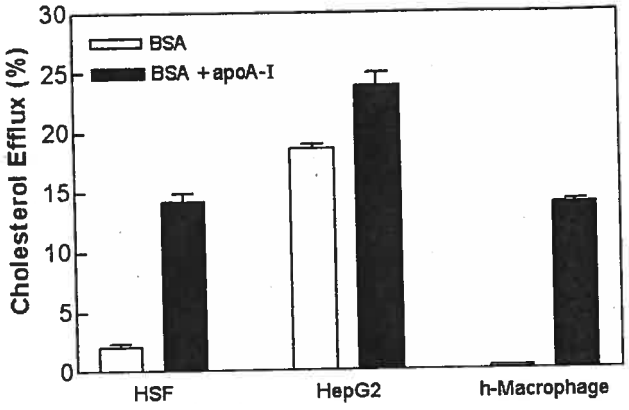

Fig. 2. Cholesterol efflux from human tissues. Human skin fibroblasts, HepG2 cells and human monocyte-derived macrophages were loaded for 24 h with cholesterol (20 μg/ml) in DMEM/BSA 2 mg/ml, and allowed to equilibrate for 24 h in DMEM/BSA 1 mg/ml. Then, medium was changed to efflux medium (DMEM/BSA 1 mg/ml ± apoA-I 10 μg/ml) and incubated for 24 h. Percentage efflux represents percent of counts in medium over counts in medium + cells. Results are expressed as means ± SD from an experiment performed in triplicate.

Cellular cholesterol levels modulate ABCA1 mRNA expression

Recent studies reported that macrophages contribute only mildly to HDL levels, we decided to concentrate on fibroblasts, a cellular model extensively used to study cholesterol efflux and Tangier disease. With the rationale of ABCA1 being important for cholesterol homeostasis in fibroblasts, we examined the regulation of ABCA1 mRNA levels in cholesterol-loaded cells. To track changes in cholesteryl content, cellular cholesterol and cholesteryl ester mass were determined by gas chromatography. Cholesterol loading resulted in dose- (Fig. 3A) and time-dependent (Fig. 3B) increases of the cellular content of free and esterified (data not shown) cholesterol, quickly followed by similar increases in ABCA1 mRNA (Fig. 3C) and protein (Fig. 3D) (glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and heat-shock protein 70 (HSP70) were used as loading controls). These data suggest a close relationship between cellular cholesterol level and ABCA1 transcription. To rule out a regulatory effect of cholesteryl esters on ABCA1 transcription, the esterification of cholesterol was blocked with avasimibe (Pfizer compound CI-1011), an inhibitor of acyl-cholesterol acetyl-transferase (ACAT) [34]. A dose of 10 μM Avasimibe abolished completely the formation of cholesteryl ester with no effect on ABCA1 mRNA levels (data not shown), thus eliminating the possibility of a regulatory effect from esterified cholesterol on ABCA1 transcription.

Cholesterol efflux downregulates ABCA1

We tested the ability of HDL_3 and apoA-I to modulate ABCA1 expression. Fig. 4 shows that efflux with

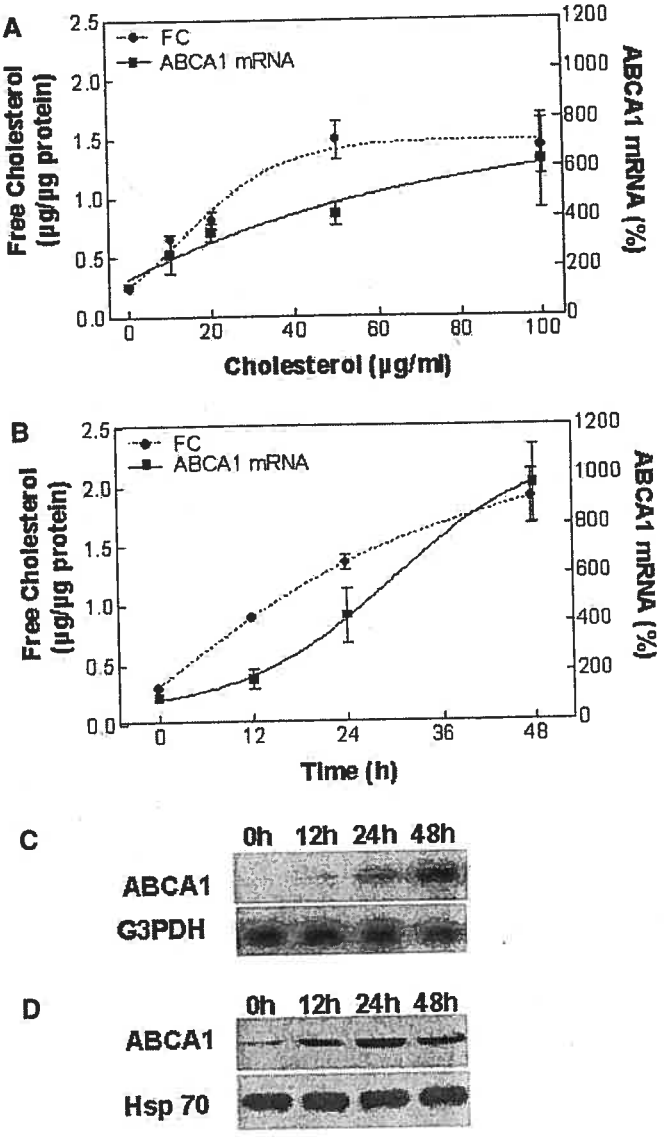

Fig. 3. Free cholesterol modulates ABCA1 mRNA in a dose and time-dependent manner. Cellular free cholesterol (FC) (dark circles) was quantified in triplicate by gas chromatography (results expressed as means ± SD). ABCA1 mRNA (dark squares) was quantified from two different blots. (A) HSF were incubated for 24 h in medium containing increasing doses of FC (0–100 μg/ml) or (B) in medium containing 20 μg/ml FC for increasing periods of time (0–48 h). Results are expressed as a percentage of the control condition. (C) Northern blot of ABCA1 mRNA controlled with G3PDH and (D) Western blot of ABCA1 protein controlled with HSP70.

HDL_3 or apoA-I (not shown) from LDL-loaded fibroblasts (Fig. 4A) decreased the abundance of the transcript by ~60% (Fig. 4B) (~45% for apoA-1, not shown). This suggests a mechanism of retroinhibition for the expression of the gene.

Cholesterol loading does not alter ABCA1 mRNA half-life

To determine whether cholesterol loading increases ABCA1 mRNA levels by increasing stability of the

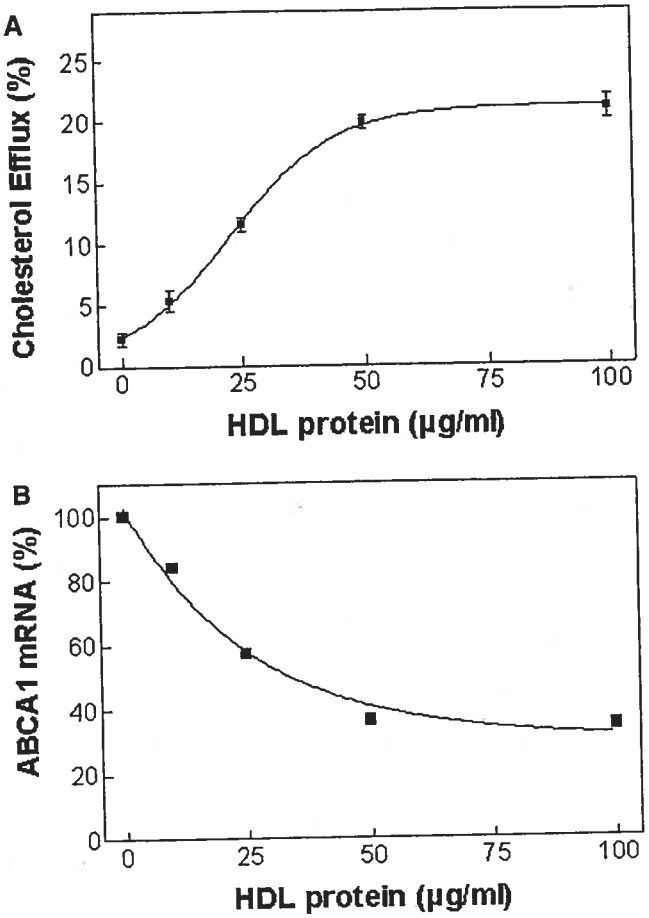

Fig. 4. Cholesterol efflux causes retroinhibition of ABCA1 transcription. LDL-loaded fibroblasts (100 µg/ml) were incubated for 24 h in DMEM/BSA containing increasing concentrations of HDL_3. (A) Cholesterol efflux was measured, or (B) total RNA was extracted and 10 µg/lane was used in a Northern blot. ABCA1 mRNA was quantified using a phosphorimager and corrected for loading with the 18S ribosomal subunit. Results are expressed as % of the control condition and are representative of two different experiments.

transcript, mRNA half-life was examined. Human skin fibroblasts were either loaded with 20 µg/ml of free cholesterol or with vehicle alone (ethanol) for 24 h. Following loading, cells were treated with DRB, an inhibitor of RNA transcription, for 0–12 h [32]. Analysis of total RNA by Northern blot revealed no significant difference between the two decay curves (Fig. 5) ($t_{1/2}$ – chol = 1.8 h; $t_{1/2}$ + chol = 2.1 h), suggesting that cholesterol loading does not alter mRNA stability and therefore the regulation is likely to be at the transcriptional level.

ABCA1 gene transcription modulation by cholesterol and hydroxysterols: independent mechanisms?

Under sterol depleted conditions, SREBP can induce transcription of genes (LDL receptor) and repress it for others (MTP) [35]. Cholesterol may modulate the ABCA1 gene via the sterol response element binding protein (SREBP) pathway or through the formation of

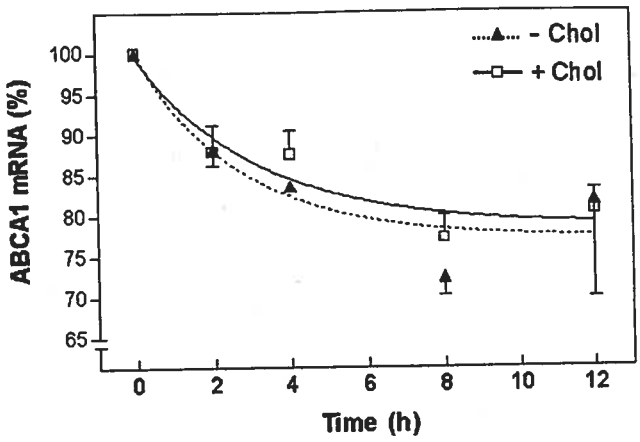

Fig. 5. Cholesterol loading does not alter ABCA1 mRNA half-life. Confluent HSF were incubated for 24 h in DMEM/BSA 2 mg/ml containing cholesterol (20 µg/ml) (open squares) or vehicle alone (closed triangles). Cells were then incubated for the indicated times in DMEM in the presence of the RNA synthesis inhibitor DRB 10 µg/ml. There was no significant difference in ABCA1 mRNA half-life between cholesterol-loaded and unloaded cells. Results represent means ± SD from three different experiments.

hydroxysterols that act as ligands for the LXR/RXR pathway. To examine whether cellular cholesterol can be converted to hydroxysterols to regulate ABCA1, we used geranylgeranyl-pyrophosphate (GGPP), an inhibitor of the interaction of the LXR/RXR complex with its target DR4 sequence [36]. It has been previously shown that GGPP, but not geranylgeraniol (GGOH) or other mevalonate metabolism pathway intermediates inhibits in vitro binding of the LXR/RXR complex to the DR4 sequences and the interaction of the dimer with their nuclear coactivator SRC-1 [37]. We first determined the optimum dose of GGPP (5 µM) required to decrease ABCA1 transcription in HSF (not shown). It is noteworthy that GGPP is unable to inhibit ABCA1 transcription induced by 9CRA (10 µM) alone (data not shown), suggesting that GGPP blocks the binding of the LXR/RXR complex to its DR4 target sequence by blocking the LXR part of the heterodimer. GGPP (5 µM) was then tested for its ability to inhibit ABCA1 transcription in cells treated with increasing concentrations of 22OH (0–5 µg/ml) or cholesterol (0–100 µg/ml) for 24 h. Fig. 6 shows that higher doses of 22OH (Fig. 6A) and cholesterol (Fig. 6B) were required to activate ABCA1 transcription when cells were incubated in presence of 5 µM GGPP. These data show that ABCA1 transcription can be competitively inhibited by the addition of GGPP, suggesting that cholesterol requires conversion to hydroxycholesterols to induce ABCA1 transcription.

Discussion

We first found that regulation of ABCA1 transcription varies among tissues. Endothelial cells, for example,

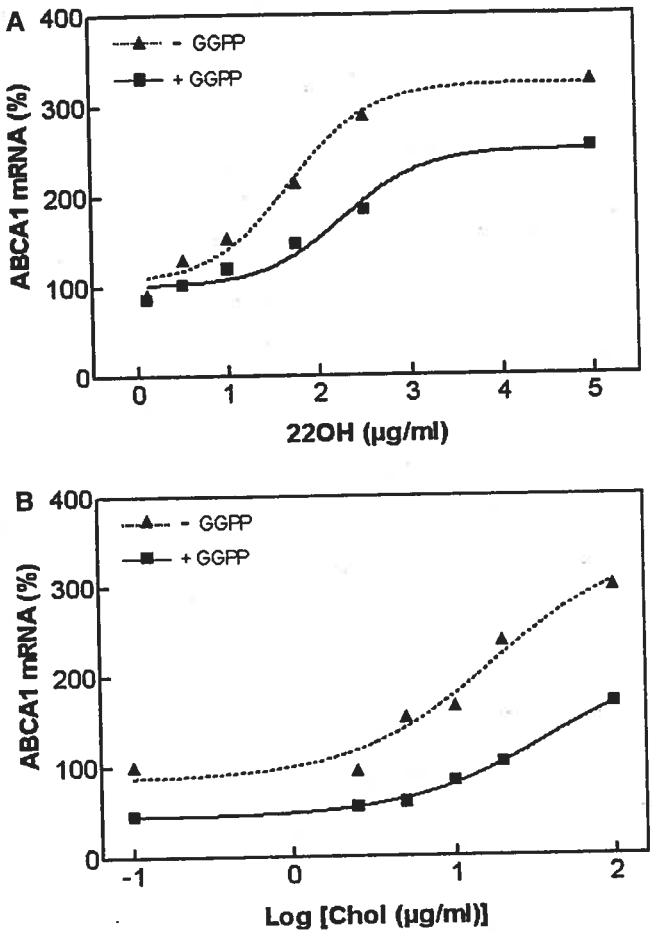

Fig. 6. Geranylgeranyl pyrophosphate inhibition of 22-(*R*)-hydroxycholesterol- and cholesterol-induced ABCA1 transcription. Competition effect of GGPP on the induction of ABCA1 transcription in HSF by (A) 22OH and (B) cholesterol. HSF were incubated for 24 h in DMEM/BSA 2 mg/ml in the presence (squares) or absence (triangles) (vehicle = methanol) of the LXR inhibitor, geranylgeranyl pyrophosphate (GGPP) 5 µM. The medium also contained increasing doses of (A) 22OH (0–5 µg/ml) or (B) cholesterol (0–100 µg/ml). ABCA1 mRNA is quantified and normalized for 28S loading. Results are expressed as a percentage of control conditions without GGPP and are representative of two different experiments. The *x*-axis in panel (B) is logarithmic for increased clarity. Results are representative of two different experiments.

do not express high levels of ABCA1 nor efflux cholesterol to apoA-I [38]. To the opposite, HepG2 cells naturally secrete cellular cholesterol carriers such as apoA-I, apoB, apoE, and bile acids, all possibly contributing to the high concentration of [^{3}H]cholesterol found in medium (Fig. 2, efflux with BSA).

We also found that 8-Br-cAMP could not significantly increase ABCA1 expression in any cell type, in contrast to what was observed in J774 mouse macrophages. Even though another group has found that cAMP analogs can induce ABCA1 transcription in immortalized HSF [39], our results are similar to those obtained in other reports [18]. Interestingly, Cavelier et al. [40] reported similar findings using a BAC containing the human ABCA1 transgene in mice. In their system, the human transgene was not regulated by cAMP analogs while the endogenous gene was. In studies on ABCA1-mediated apoA-I binding [14] and cholesterol efflux [18], cAMP analogs are often used to induce expression of ABCA1 mRNA in mouse macrophages. Some authors propose the cAMP induction pathway as a potential target for therapeutic purposes [41]. However, our results raise the question of the relevance of cAMP as a physiological inducer of ABCA1 in humans. First, no study has ever demonstrated any correlation between endogenous cAMP increases and ABCA1 induction. Second, as our results show that cAMP does not induce ABCA1 expression, we conclude that cAMP is not a universal inducer of ABCA1 transcription. This does not exclude however post-translational regulation of ABCA1 by cAMP through protein phosphorylation [42].

Monocyte-derived macrophages and HSF show similar regulation patterns. However we decided to study the cholesterol homeostasis in fibroblasts, a well-known model to study Tangier disease. We found that cholesterol loading transcriptionally (Fig. 5) modulates the abundance of ABCA1 mRNA (Figs. 3A–C) and protein (Fig. 3D) in HSF. Using an ACAT inhibitor also found that cholesteryl esters do not modulate ABCA1 transcription. Cholesterol efflux to HDL_3 or apoA-I also decreased mRNA abundance, suggesting a close relationship between cellular sterol levels and transcription.

To determine the mechanism by which cholesterol induces ABCA1 transcription in HSF, we found that low doses of hydroxysterols modulate the ABCA1 gene in HSF and that an inhibitor of LXR, GGPP, decreases ABCA1 basal and 22OH-induced transcription. This indicates that cholesterol conversion to a LXR ligand occurs in HSF and confirms an important role of the LXR/RXR pathway in transcription in HSF. The LXR nuclear factor is activated by hydroxysterol ligands, but not by cholesterol [43]; its endogenous ligand in HSF remains to be determined. A recent report suggests that 27-hydroxycholesterol is a good candidate ligand as cholesterol loading of skin fibroblasts from patients with cerebrotendinous xanthomatosis (cholesterol 27-hydroxylase deficiency) failed to increase ABCA1 transcription in HSF [44]. Thus it seems that hydroxysterols are acting as a proxy sensor for the presence of cholesterol. However, it remains to be determined whether or not cholesterol could modulate ABCA1 by mechanisms independent of conversion to hydroxysterols. Dual mechanisms of regulation by cholesterol and hydroxysterols are possible. One example of this is the promoter of the CETP gene that contains SRE elements [45] as well as LXR/RXR consensus sequences [46]. In our case, cellular cholesterol might modulate ABCA1 via three

pathways: first, a direct effect on the ABCA1 promoter region, mediated via SRE-like sequences; second, through the inhibition of a transcriptional repressor, such as ZNF-202 [47], or third, through the inhibition of HMG CoA reductase and an increase in PPAR activity. It has been suggested that an increase in macrophage PPARα activity induces ABCA1 transcription [48] and that PPARγ agonists enhance LXR transcription which subsequently increase ABCA1 mRNA [49].

GGPP inhibits in vitro binding of the LXR/RXR complex to its target DR4 sequence [36] and was shown to decrease LXR/RXR-mediated induction of the CYP7A1 promoter activity in HepG2 cells [50]. However, GGPP is also a substrate for the prenylation of proteins, especially small G-proteins [51]. In their study, Gan et al. [37] used an inhibitor of geranylgeranyl transferase, the enzyme transferring GGPP onto Rho proteins, causing a subsequent increase in ABCA1 transcription. They conclude that the prenylation of small G-proteins with GGPP is also involved in the regulation of ABCA1 transcription. However, at the dose selected here, the effect of GGPP is inhibitory and therefore likely to be due to direct inhibition of LXR.

ABCA1 is differentially regulated in human cell types. Even though endothelial cells constitute an important tissue for atherosclerosis, low level of ABCA1 and absence of cholesterol efflux to delipidated apoA-I suggests a minimal (if no) contribution to formation of pre-β particles [38]. Also, macrophages contribute only mildly to HDL levels [24].

Our study revealed that sterols modulate ABCA1 transcription in human skin fibroblasts. We hypothesize that conversion of cholesterol to hydroxycholesterol contributes to this regulation, but cannot exclude an independent action of free cholesterol. Fibroblasts constitute an important tissue of the body and might contribute importantly to the early steps of HDL formation. However, the importance of the liver in the generation of poorly lipidated apoA-I particles is well established in virtue of its ability to synthesize apoA-I and ABCA1. Studies on cholesterol efflux in Tangier fibroblasts have shown that absence of functional ABCA1 causes low efflux, and consequently a hypercatabolism of immature particles [52]. We have previously shown that plasma HDL-C levels correlate with cellular cholesterol efflux and that PKA can modulate the ABCA1-dependent efflux at the post-transcriptional level [42]. Taken together, these findings open the possibility of pharmacological tissue-specific modulation of the ABCA1 efflux pathway for therapeutic purposes.

Acknowledgments

A doctoral research award from the Heart and Stroke Foundation of Canada supports M. Denis. The work is supported by CIHR Grant MOD 15042 and CHIR-Rx&D Grant DOP 48045. J. Genest holds a CIHR-Novartis chair at McGill University.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 9, Issue of February 27, pp. 7384–7394, 2004

Printed in U.S.A.

Molecular and Cellular Physiology of Apolipoprotein A-I Lipidation by the ATP-binding Cassette Transporter A1 (ABCA1)*

Received for publication, June 30, 2003, and in revised form, December 1, 2003
Published, JBC Papers in Press, December 4, 2003, DOI 10.1074/jbc.M306963200

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The dynamics of ABCA1-mediated apoA-I lipidation were investigated in intact human fibroblasts induced with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid (stimulated cells). Specific binding parameters of ^{125}I-apoA-I to ABCA1 at 37 °C were determined: K_d = 0.65 μg/ml, B_{max} = 0.10 ng/μg cell protein. Lipid-free apoA-I inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than pre-β_1-LpA-I, reconstituted HDL particles r(LpA-I), or HDL_3 (IC_{50} = 0.35 ± 1.14, apoA-I; 1.69 ± 1.07, pre-β_1-LpA-I; 17.91 ± 1.39, r(LpA-I); and 48.15 ± 1.72 μg/ml, HDL_3). Treatment of intact cells with either phosphatidylcholine-specific phospholipase C or sphingomyelinase affected neither ^{125}I-apoA-I binding nor ^{125}I-apoA-I/ABCA1 cross-linking. We next investigated the dynamics of apoA-I lipidation by monitoring the kinetic of apoA-I dissociation from ABCA1. The dissociation of ^{125}I-apoA-I from normal cells at 37 °C was rapid ($t_{1/2}$ = 1.4 ± 0.66 h; n = 3) but almost completely inhibited at either 15 or 4 °C. A time course analysis of apoA-I-containing particles released during the dissociation period showed nascent apoA-I-phospholipid complexes that exhibited α-electrophoretic mobility with a particle size ranging from 9 to 20 nm (designated α-LpA-I-like particles), whereas lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells was unable to form such particles. These results demonstrate that: 1) the physical interaction of apoA-I with ABCA1 does not depend on membrane phosphatidylcholine or sphingomyelin; 2) the association of apoA-I with lipids reduces its ability to interact with ABCA1; and 3) the lipid translocase activity of ABCA1 generates α-LpA-I-like particles. This process plays *in vivo* a key role in HDL biogenesis.

Apolipoprotein (apo)[1] A-I binding to the extracellular domain of ABCA1 results in the activation of apoA-I lipidation, a key step in reverse cholesterol transport (RCT) process, one of the several proposed mechanisms by which HDL may protect against atherosclerotic vascular disease (1–3).

The molecular interaction of apoA-I with ABCA1 promotes cholesterol efflux from peripheral cells and macrophages and is critical for the initial formation of HDL particles (1). The importance of ABCA1 in the lipidation of apoA-I has been strikingly demonstrated by the identification of mutations at the *ABCA1* gene locus as the molecular defect of Tangier Disease (TD) and Familial HDL Deficiency (FHD) (4, 5). These patients are characterized by extremely low HDL-cholesterol levels, caused by inadequate transport of cellular cholesterol and phospholipids to the extracellular space, leading to hypercatabolism of lipid-poor nascent HDL particles (6).

ApoA-I has been shown to interact with many proteins including high-density lipoprotein-binding protein (HBP, vigilin), HB2 (7), annexin I, annexin VII (8), fibronectin, collagen I (9, 10), and the human β-chain of ATP synthase (11). However, the physiological significance of these interactions remains unknown. On the other hand, it is well established that apoA-I binds to the scavenger receptor class B type I (SR-BI) (12), which participates in selective uptake of HDL-derived cholesteryl esters, but so far no role for SR-BI in apoA-I-mediated lipid efflux has been found.

Although several studies have suggested a molecular interaction between apoA-I and ABCA1 at the cell surface (13–15), the role of ABCA1 as a candidate apoA-I receptor is still a matter of debate. At least two different mechanisms are proposed for this interaction. First, it is reported that a direct protein-protein interaction occurs between apoA-I and ABCA1 on the basis of chemical cross-linking experiments (13). A second hypothesis has been proposed suggesting an interaction between apoA-I and lipid domains in the cell membranes formed by the phospholipid translocase activity of ABCA1 (14). Indeed, studies by Remaley *et al.* (16, 17) have shown that a majority of the plasma apolipoproteins containing lipophilic class A amphipathic helices can also promote lipid efflux and bind to ABCA1. Furthermore, the amphipathic helix was found to be a key structural motif for peptide-mediated lipid efflux from ABCA1.

Without knowledge of specific binding parameters of apoA-

* This work was supported by Grants MOP 15042 from the Canadian Institutes of Health Research (CIHR) and the Heart and Stroke Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a personnel award from the Heart and Stroke Foundation of Canada.

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[1] The abbreviations used are: apo, apolipoprotein; PAGGE, polyacrylamide non-denaturing gradient gel electrophoresis; ABCA1, ATP-binding cassette AI; BSA, bovine serum albumin; CETP, cholesteryl ester transfer protein; FHD, Familial HDL deficiency; HDL, high density lipoprotein; H-TGL, hepatic lipase; LCAT, lecithin: cholesterol acyl transferase; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; r(LpA-I), reconstituted HDL particles; RCT, reverse cholesterol transport; SM-ase, sphingomyelinase; SM, sphingomyelin; SR-BI, scavenger receptor class B type I; TD, Tangier disease; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FC, free cholesterol; PL, phospholipids; TLC, thin layer chromatography.

I-containing particles to ABCA1, it is not possible to predict whether ABCA1 might function as a significant receptor for apoA-I in the presence of other apolipoproteins, which demonstrate affinity for the same protein. A recent study by Basso *et al.* (18) demonstrating that the hepatic expression of ABCA1 is an important source of plasma HDL-C has stimulated our interest for apoA-I lipidation in peripheral cells. In the present study, experiments were directed at defining the mechanism by which apoA-I is lipidated by ABCA1 and how the formation of the apoA-I/ABCA1 complex can be affected by apoA-I conformation within discoidal and spherical HDL particles, by specific hydrolysis of plasma membranes phospholipids, or by naturally occurring mutants of ABCA1. In addition, the dynamics of apoA-I lipidation were investigated by determining the kinetic parameters of apoA-I/ABCA1 dissociation and the characterization of apoA-I-containing particles generated during this process.

EXPERIMENTAL PROCEDURES

Patient Selection—For the present study, we selected fibroblasts from 3 normal control subjects and 1 patient with TD (homozygous for Q597R at the *ABCA1* gene). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Center. Separate consent forms for blood sampling, DNA isolation, and skin biopsy were provided.

Cell Culture—Human skin fibroblasts were obtained from 3.0-mm punch biopsies of the forearm of patients and healthy control subjects and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum.

Human Plasma ApoA-I—Purified plasma apoA-I (Biodesign) was resolubilized in 4 M guanidine HCL and dialyzed extensively against Tris buffer, (10 mM Tris, 150 mM NaCl; pH 8.2). Freshly resolubilized apoA-I was used within 48 h.

ApoA-I Binding Assay—ApoA-I was iodinated with ^{125}I by IODOGEN® (Pierce) to a specific activity of 800–2500 cpm/ng apoA-I. Cells were grown on 24-well plates and were stimulated or not with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Cells were then incubated at 37 °C with ^{125}I-apoA-I in DMEM/BSA (1 mg/ml) as specified for each experiment in the presence or absence of a 30-fold excess of unlabeled apoA-I, to subtract the nonspecific binding. The cells were then washed rapidly two times with ice-cold PBS/BSA, two times with cold PBS and lysed with 0.1 N NaOH. The amount of bound iodinated ligand was determined by γ-counting.

Chemical Cross-linking and Immunoprecipitation Analysis—Chemical cross-linking was performed as described by Wang *et al.* (19) with a minor modification. Fibroblasts were grown to confluence in 100-mm diameter dishes and then stimulated or not with 2.5 μg/ml 22(R)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h in DMEM/BSA. Cells were incubated in the presence or absence of either 3 μg/ml of unlabeled apoA-I or 10 μg/ml of ^{125}I-apoA-I in DMEM/BSA for 1 h at 37 °C. Cells were then placed on ice for 15 min and washed three times with PBS. DSP (cross-linker agent) was dissolved immediately before use in dimethyl sulfoxide (Me_2SO) and diluted to 500 μM with PBS. 8 ml of DSP solution was added in each well. Cells were then incubated at room temperature for 1 h; the medium was removed, and the cells were washed twice with PBS. Cells were lysed at 4 °C with IP buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton X-100 (Invitrogen), and the suspension was allowed to stand for 30 min at 4 °C in presence of a protease inhibitor mixture (Roche Diagnostics). ApoA-I/ABCA1 complex was immunoprecipitated with an affinity-purified polyclonal anti-ABCA1 antibody (Novus Biologicals) as previously described (20). After SDS-gel electrophoresis either apoA-I or ABCA1 were detected by immunopurified polyclonal anti-apoA-I antibody (Biodesign) or affinity-purified human anti-ABCA1 antibody (Novus). The presence of labeled ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography using XAR-2 Kodak film.

Quantitative Cross-linking of ApoA-I to ABCA1—Fibroblasts were grown to confluence in 100-mm diameter dishes and then stimulated for 20 h. Cells were incubated at 37 °C for 1 h in the presence or absence of 5 units/ml PC-PLC or 0.4 units/ml SM-ase. After washing to remove phospholipases, cells were incubated with 10 μg/ml of ^{125}I-apoA-I (1500–2500 cpm/ng) in the presence or absence of a 20-fold excess of unlabeled apoA-I. Cells were then placed on ice for 15 min and washed three times with PBS, and then cross-linking with DSP was performed as described above. Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 μg of total protein) were incubated with 10 μl of affinity-purified human anti-ABCA1 antibody for 20 h at 4 °C, followed by the addition of protein A bound to Sepharose (30 μl) as we have described previously (21). The amount of bound iodinated apoA-I to ABCA1 in the immunoprecipitates was determined by γ-counting. ABCA1 mutant (Q597R) was used as a negative control.

Dissociation of Specifically Bound ^{125}I-ApoA-I from Intact Cells—Fibroblasts were grown to near confluence in 24-well plates and then stimulated with 2.5 μg/ml 22(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h in DMEM/BSA. The cells were incubated for 2 h at 37 °C with 10 μg/ml of ^{125}I-apoA-I in the presence of 1 mg/ml BSA. For nonspecific binding determination, cells were incubated with a 30-fold excess of unlabeled apoA-I. After washing to remove unbound ^{125}I-apoA-I, 0.5 ml of DMEM was added, and the plates were immediately incubated at 37 °C, 15 °C, or 4 °C for the indicated times. The medium was then collected, cells were lysed in 0.1 N NaOH, and the radioactivity in the medium and in the cells was determined by γ-counting.

Cellular Lipid Efflux and Lipid Labeling—Phospholipid and cholesterol efflux were determined as previously described (3) with minor modifications. Briefly, 50,000 cells were seeded in 12-well plates. At mid-confluence, the cells were labeled with 0.2–5 μCi/ml [^{3}H]choline (PerkinElmer Life Sciences) or 0.2–5 μCi/ml [^{3}H]cholesterol (PerkinElmer Life Sciences) for 48 h. At confluence, cells were cholesterol-loaded (20 μg/ml) for 24 h. During a 24 h equilibration period, cells were stimulated or not with 2.5 μg/ml of 22(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Phospholipid or cholesterol efflux were determined at either 2 or 24 h with 10 μg/ml apoA-I. Cellular lipid efflux was determined as follow: ^{3}H cpm in medium/(^{3}H cpm in medium + ^{3}H cpm in cells); the results were expressed as percent of total radiolabeled phospholipids or cholesterol. Cell phospholipids were also labeled with [^{32}P]orthophosphate as follows: fibroblasts from control subject were grown to confluence in 100-mm or 150-mm diameter dishes and were incubated for 72 h with 300–1500 μCi of [^{32}P]orthophosphate mixed with DMEM. The cells were stimulated as described above before incubation with lipid-free apoA-I as specified for each experiment.

Separation of Lipoproteins by Two-dimensional Non-denaturing Gradient Gel Electrophoresis—ApoA-I-containing particles were separated by two-dimensional-PAGGE, as previously described (22, 23). Briefly, samples (30–100 μl) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4 °C) and in the second dimension (according to the size) by 5–23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4 °C). Iodinated high molecular weight protein mixture (7.1–17.0 nm, Amersham Biosciences) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4 °C) onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences). ApoA-I-containing particles were detected by incubating the membranes with immunopurified polyclonal anti-apoA-I antibody (Biodesign) labeled with ^{125}I. The presence of labeled ^{125}I-apoA-I or ^{32}P-phospholipids were directly detected by autoradiography using XAR-2 Kodak film.

Preparation of Reconstituted HDL Particles (rLpA-I)—Complexes comprising apoA-I, POPC, and cholesterol were prepared using the sodium cholate dialysis method (24). ApoA-I/POPC/cholesterol molar ratio of 1:100:5 was used in this experiment. r(LpA-I) particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 50,000, Amicon) to discard any lipid-free apoA-I or proteolytic peptides. ApoA-I-lipid complex formation was verified by analysis with two-dimensional-PAGGE.

Pre-β_1-LpA-I Purification from Plasma—Pre-β_1-LpA-I was purified from freshly drawn venous blood under nondenaturing conditions as described by Kunitake *et al.* (25) with the following modifications. Typically, blood is drawn into a tube containing 1 mM sodium EDTA, 0.02% NaN_3, 2 mM DTNB, and cooled immediately on ice. Plasma is separated by low speed centrifugation (1,800 × *g*, 30 min) and aliquots (20 ml) were subjected to human immunopurified anti-apoA-I antibody (12171–21A, Genzyme Corp)-coupled Sepharose column (23, 26). ApoA-I-containing fractions were then dialyzed and concentrated. Samples were separated by agarose gel electrophoresis, and the pre-β-migrating region was excised out. Agarose gel pieces containing the pre-β-migrating region were placed at the top of 3–26% non-denaturing gradient gels, as previously described (27). An immunoblot of apoA-I-containing lipoproteins separated by two-dimensional-PAGGE gels was used as a template to localize pre-β_1-LpA-I particles, which are recovered from the gels by electroelution. Pre-β_1-LpA-I particles were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, MWCO 50,000,

Amicon) to discard any lipid-free apoA-I or proteolytic peptides. The integrity of isolated plasma pre-β_1-LpA-I fraction was verified by two-dimensional-PAGGE.

RESULTS

In the present study, we have examined the binding of ^{125}I-apoA-I to ABCA1 in normal cultured human fibroblasts. To determine the specific binding of ^{125}I-apoA-I to ABCA1, binding studies were performed in fibroblasts in which ABCA1 was induced with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid (stimulated cells), as well as in unstimulated cells. As shown in Fig. 1*A*, a marked and consistent increased binding of ^{125}I-apoA-I to stimulated cells was measured. However, significant binding was also observed in unstimulated cells. This is presumably due to basal level of ABCA1 expression and the presence of other apoA-I binding sites at the cell surface. We have not been able to detect any SR-BI receptor presence in fibroblasts as compared with hepatocytes as examined by gel electrophoresis of cellular membranes fraction followed by immunoblotting with an anti-SR-BI antibody (data not shown). The specific binding curve and the binding parameters K_d and B_{max} for apoA-I/ABCA1 interactions were determined by subtracting the binding values for the unstimulated cells from the corresponding values from stimulated cells. In the present binding assay apoA-I binds to ABCA1 (ABCA1 specific) with relatively high affinity (K_d = 0.65 ± 0.20 μg/ml), and the binding was saturable (B_{max} = 0.10 ± 0.05 ng/μg cell protein) (Fig. 1*A*). Maximum specific binding of apoA-I to ABCA1 was reached in less than 30 min and remained constant for the remaining 2 h of the experiment (data not shown). To ensure that the binding parameters obtained reflect specific increased of ^{125}I-apoA-I/ABCA1 association in stimulated cells, the cross-linking of apoA-I to ABCA1 was examined. As shown in Fig. 1*B*, apoA-I forms a complex with ABCA1. Furthermore, stimulation of cells lead to an increase of both cellular ABCA1 expression and apoA-I/ABCA1 cross-linking compared with unstimulated cells. At the same time, phospholipid and cholesterol efflux were increased in stimulated cells, as shown in Fig. 1*C*. In order to verify that the specific association of ^{125}I-apoA-I with ABCA1 was dependent on the temperature, stimulated cells were incubated with 10 μg/ml of ^{125}I-apoA-I for 2 h at either 37 °C, 20 °C or 4 °C, and then specific ^{125}I-apoA-I cell association was determined as described above. Association with ^{125}I-apoA-I-stimulated cells showed remarkable temperature dependence (100 ± 2%, 29 ± 4% and 13 ± 2%; 37 °C, 20 °C and 4 °C, respectively). Results are expressed as percent of the incubation at 37 °C (100%).

It is well established that the conformation of apoA-I within HDL particles is affected by its association with lipid molecules. It was therefore of interest to determine whether apoA-I conformation/organization within particles would affect its interaction with ABCA1. Competition assays were performed to determine the ability of pre-β_1-LpA-I, as well as discoidal reconstituted HDL r(LpA-I) and spherical HDL particles (HDL_3), to compete for the binding of ^{125}I-apoA-I to ABCA1 in stimulated cells. As shown in Fig. 2*A*, lipid-free apoA-I inhibited the binding of ^{125}I-apoA-I to ABCA1 more efficiently than either isolated plasma pre-β_1-LpA-I, reconstituted HDL particles r(LpA-I) (11–12 nm of diameter), or native HDL_3 (IC_{50} = 0.35 ± 1.14, apoA-I; 1.69 ± 1.07, pre-β_1-LpA-I; 17.91 ± 1.39, r(LpA-I); and 48.15 ± 1.72 μg/ml, HDL_3). Control experiments were conducted to examine whether the apparent decrease in cell binding of the labeled apoA-I may be due to the ^{125}I-apoA-I binding to different competitors particles instead of the cells. An experiment was therefore carried out in which either r(LpA-I) or HDL_3 particles were incubated with ^{125}I-apoA-I under similar conditions used for apoA-I binding assay, and then the samples were separated by fast protein liquid chromatography (FPLC). No significant amount of ^{125}I-apoA-I was found associated with r(LpA-I) or HDL_3 (data not shown), supporting our results shown in Fig. 2*A*. To verify the integrity of competitors particles, either isolated pre-β_1-LpA-I, lipid-free apoA-I, r(LpA-I), or plasma were separated by two-dimensional-PAGGE and apoA-I was detected with immunopurified polyclonal anti-apoA-I antibody labeled with ^{125}I, as shown in Fig. 2*B*.

Because the lipid binding characteristics of apoA-I have been proposed to be important in the apoA-I/ABCA1 interaction (14, 15, 28), the question was raised whether the binding of apoA-I to ABCA1 was dependent on the presence of lipids at the cell surface. Stimulated cells were incubated for 60 min at 37 °C in the presence or absence of either 5 units/ml phosphatidylcholine-specific phospholipase C (PC-PLC) or 0.4 units/ml sphingomyelinase (SM-ase). To assess how effectively phospholipids were removed by phospholipases treatment, the cells were labeled with [^{3}H]choline and the lipids separated by TLC and counted. PC-PLC and SM-ase treatment digested greater than 65% of phosphatidyl [^{3}H]choline and 80% of [^{3}H]sphingomyelin, respectively. Cells were then incubated with 10 μg/ml of ^{125}I-apoA-I for 2 h at 37 °C and specific ^{125}I-apoA-I binding was determined as described above. As shown in Fig. 3*A*, no significant effect of phospholipases treatment on the ^{125}I-apoA-I binding level was observed. In order to further verify that the interaction of apoA-I with ABCA1 was not dependent on the presence of plasma membrane phosphatidylcholine or sphingomyelin, we determined whether the cross-linking of apoA-I to ABCA1 could be affected by phospholipases treatment. Quantitative cross-linking of apoA-I to ABCA1 was performed as described in "Experimental Procedures." As shown in Fig. 3*B* (*lower panel*), treatment of intact cells with phospholipases did not affect significantly ^{125}I-apoA-I cross-linking to ABCA1. The presence of a 20-fold excess of unlabeled apoA-I (200 μg/ml) reduced the cross-linking of ^{125}I-apoA-I to ABCA1 by 78% of control. In addition, ABCA1 mutant (Q597R) that has been shown previously to not cross-link to apoA-I (13) was used as a negative control for the present experiment and showed no binding or cross-linking to ABCA1 (Fig. 3, *A* and *B*). To ensure that the immunoprecipitates contained only ^{125}I-apoA-I/ABCA1 complex, immunoprecipitated samples were analyzed by 4–22.5% SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3*B* (*upper panel*), only ^{125}I-apoA-I/ABCA1 complex was detected in immunoprecipitated samples. Also, to rule out the possibility that treatment with phospholipases might induce membrane aggregation or affect ABCA1 protein structure, which may trap apoA-I and result in nonspecific cross-linking, we examined the effect of phospholipases treatment on the cross-linking of ^{125}I-apoA-I to ABCA1 mutant (Q597R). As shown in Fig. 3*B* (*upper panel*), ^{125}I-apoA-I did not cross-link to Q597R mutant whether treated with PC-PLC, SM-ase, or left intact.

We initially hypothesized that any specific apoA-I dissociation from ABCA1 would be associated with a significant increase in apoA-I lipidation state. To better understand the mechanism by which apoA-I was lipidated by ABCA1, the kinetics of the dissociation of apoA-I from ABCA1 were investigated in stimulated cells. Fig. 4*A* depicts the time course of the dissociation of bound ^{125}I-apoA-I from stimulated normal cells at 37 °C. The dissociation of ^{125}I-apoA-I from ABCA1 at 37 °C was rapid ($t_{1/2}$ = 1.4 ± 0.66 h; n = 3). In contrast, ^{125}I-apoA-I dissociation from ABCA1 was almost completely inhibited at either 4 or 15 °C (Fig. 4*A*). Practically all radioactivity that disappeared from the cell surface appeared as intact ^{125}I-apoA-I in the medium (more than 95% of the radioactivity

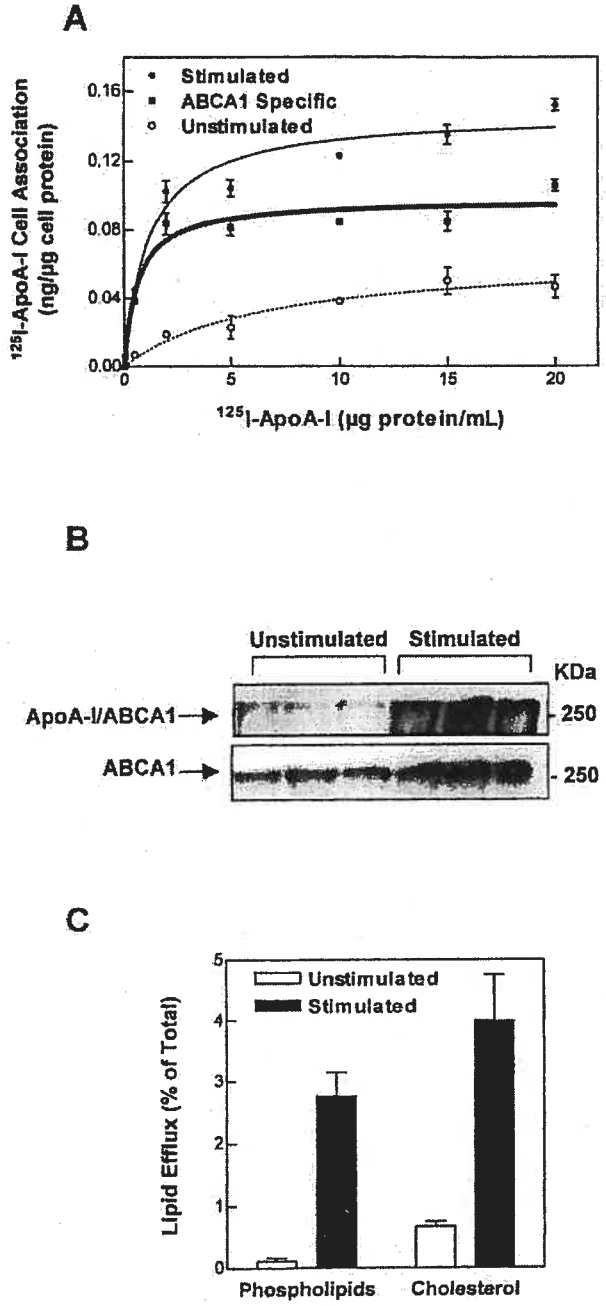


FIG. 1. **Effect of 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid on ^{125}I-apoA-I cell association, apoA-I/ABCA1 complex formation and cellular lipid efflux.** *A*, normal control fibroblasts were plated in 24-well plates and stimulated or not with 2.5 µg/ml 22(*R*)-hydroxycholesterol and 10 µM 9-*cis*-retinoic acid for 20 h. Cells were then incubated for 2 h at 37 °C with increasing amounts of ^{125}I-apoA-I (0, 2.5, 5, 10, 15, 20 µg/ml). Nonspecific binding was determined for both stimulated and unstimulated cells in the presence of a 30-fold excess of unlabeled apoA-I. The specific binding curve (ABCA1-specific) was determined by subtracting the binding values for the unstimulated cells from the corresponding values for stimulated cells. Binding parameters of ^{125}I-apoA-I to ABCA1 were analyzed using Graph Pad Prism 4.00 software. *B*, stimulated and unstimulated fibroblasts were incubated with 3 µg/ml apoA-I at 37 °C for 1 h. Cells were washed two times with cold PBS and exposed to the DSP cross-linker for 1 h at room temperature. ApoA-I/ABCA1 complexes were immunoprecipitated with an anti-ABCA1 antibody and run on 6% SDS-PAGE. ApoA-I associated with ABCA1 (*upper panel*) or ABCA1 itself (*lower panel*) were detected by immunoblotting with an anti-apoA-I antibody or an anti-ABCA1 antibody. *C*, stimulated and unstimulated normal cells were radiolabeled with either [^{3}H]cholesterol or [^{3}H]choline and incubated with 10 µg/ml of apoA-I at 37 °C for 2 h. Phospholipid and cholesterol efflux were determined as described under "Experimental Procedures." *Bars* represent mean ± S.D. of an experiment performed in triplicate.

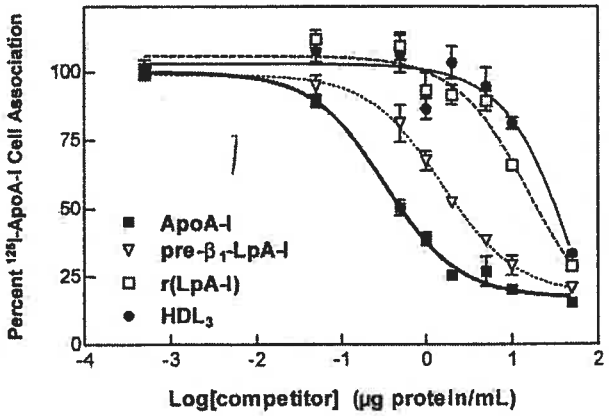
released to the medium was precipitated by 10% trichloroacetic acid).

To further investigate the relationship between apoA-I dissociation from ABCA1 and apoA-I-mediated cellular cholesterol efflux in our stimulated cell culture system, the kinetics of cholesterol efflux in stimulated cells was determined in the presence of 10 µg/ml of apoA-I (saturating binding concentrations). As shown in Fig. 4*B*, apoA-I-mediated cholesterol efflux reached saturation after a 16-h incubation period. In addition, to ensure that cholesterol efflux was ABCA1-dependent in our cell culture system, apoA-I-mediated cholesterol efflux from ABCA1 mutant (Q597R) cells was also monitored.

In order to investigate the nature of apoA-I-containing particles generated by ABCA1 activity, stimulated cells from either normal or from TD (Q597R) subjects in 100 mm diameter dishes were incubated with 10 µg/ml of ^{125}I-apoA-I in 8 ml of DMEM for 24 h at 37 °C. The medium was concentrated and ^{125}I-apoA-I-containing particles were separated by two-dimensional-PAGGE. As shown in Fig. 5 (*panel B*), apoA-I-containing particles generated by stimulated normal cells exhibited α-electrophoretic mobility with a particle diameter ranging from 9 to 20 nm, however, a significant amount of apoA-I was detected in the pre-β-region. In contrast, lipid-free apoA-I incubated with stimulated mutant Q597R cells was unable to form such particles (*panel C*), which had a molecular diameter and charge similar to the lipid-free apoA-I incubated in the same conditions without cells (*panel A*).

To further characterize apoA-I-containing particles released specifically from ABCA1 during the dissociation period, stimulated cells in 150-mm diameter dishes were incubated with 10 µg/ml ^{125}I-apoA-I for 2 h at 37 °C. After washing to remove unbound ^{125}I-apoA-I, 15 ml of DMEM was added, and the plates were immediately incubated at 37 °C for 1.4, 8, and 24 h. The medium was concentrated and ^{125}I-apoA-I-containing particles were electrophoretically separated by two-dimensional-PAGGE. As shown in Fig. 6, ^{125}I-apoA-I incubated for 24 h without cells had a pre-β electrophoretic mobility with a molecular diameter of 7.1 nm (*panel A*). However, apoA-I-containing particles dissociated from normal stimulated cells at either 1.4, 8, or 24 h exhibited α-electrophoretic mobility with a particle size ranging from 9 to 20 nm (designated α-LpA-I-like particles) (*panels B–D*, respectively). Both the charge and size of these nascent particles are stable over a 24-h dissociation period. We next examined whether the α-electrophoretic mobility of LpA-I-like particles may be caused by specific phospholipid composition. Cells were first labeled with [^{32}P]orthophosphate, then stimulated and incubated with 10 µg/ml of unlabeled apoA-I for 2 h at 37 °C. Dissociated ^{32}P-phospholipidated apoA-I was analyzed by two-dimensional-PAGGE as described above. As shown in Fig. 6 (*panels E–G*), ^{32}P-phospholipidated apoA-I co-localized with the majority of ^{125}I-α-LpA-I-like particles (*panels B–D*). We next determined the relative phospholipid composition of α-LpA-I-like particles. The medium containing α-LpA-I-like particles at different time was concentrated, dialyzed and apoA-I-containing particles were immunoprecipitated with an anti-apoA-I antibody. The ^{32}P-labeled phospholipids sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), and phosphatidylinositol (PI) were extracted from immunoprecipitated medium, then separated in triplicate on TLC and quantified by phosphorimager. Percent phospholipid composition of α-LpA-I-like particles was: SM, 16 ± 1%; PC, 51 ± 1%; PE, 15 ± 0.6%; LPC, 4.4 ± 1.3% and PI, 14 ± 0.2%. The ratio of phospholipid species present in α-LpA-I-like particles did not change significantly at either 1.4 h, 8 h or 24 h dissociation period (data not shown).

A



Competitor	IC_{50} (μg protein/mL)
ApoA-I	0.35 ± 1.14
pre-β_1-LpA-I	1.69 ± 1.07
r(LpA-I)	17.91 ± 1.39
HDL_3	48.15 ± 1.72

B

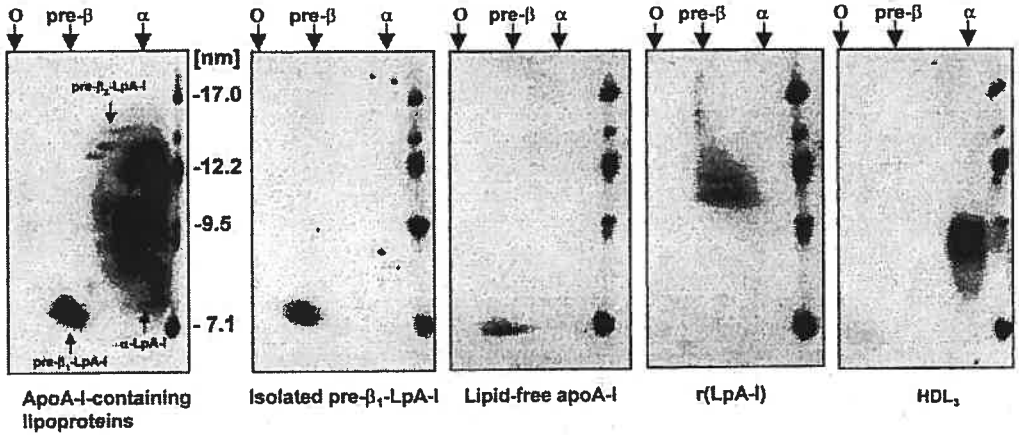


FIG. 2. **Ability of pre-β_1-LpA-I particles, reconstituted HDL particles r(LpA-I) and native HDL_3 to interact with ABCA1.** *A*, normal cells were plated in 24-well plates and stimulated for 20 h. Cells were then incubated with 2 μg/ml of ^{125}I-apoA-I for 2 h at 37 °C with increasing amounts of either plasma isolated pre-β_1-LpA-I, reconstituted HDL r(LpA-I), native HDL_3, and unlabeled apoA-I (0, 0.05, 0.5, 1, 2, 5, 10, 50 μg protein/ml). Cells were then washed rapidly three times with ice-cold PBS/BSA and then PBS alone. ^{125}I-apoA-I associated with cells was determined as described under "Experimental Procedures." The values shown represent the mean ± S.D. from triplicate wells. The 100% of control value measured in the absence of competitors was 0.8 ng of apoA-I/μg cell protein. Similar results were obtained in four independent experiments. Values of IC_{50} shown were determined using the Graph Pad Prism 4.00 software. *B*, either plasma isolated pre-β_1-LpA-I, reconstituted HDL r(LpA-I), native HDL_3 or plasma were separated by two-dimensional-PAGGE and apoA-I was detected with immunopurified polyclonal anti-apoA-I antibody labeled with ^{125}I. Molecular size markers are indicated on the *right side* of each gel.

DISCUSSION

In view of the importance of ABCA1 in the regulation of plasma HDL cholesterol (1, 3, 18), we investigated the molecular and physiological mechanisms of ABCA1-dependent apoA-I lipidation in fibroblasts as a model for peripheral cells. Consistently with an earlier study by Remaley *et al.* (16), we show that specific binding parameters of ^{125}I-apoA-I to ABCA1 could be measured in ABCA1-stimulated cells (Fig. 1*A*). The specificity of ^{125}I-apoA-I binding to ABCA1 was supported by experiments showing that apoA-I forms a complex with ABCA1 in unstimulated cells, and this effect was markedly enhanced in stimulated cells (Fig. 1*B*). Moreover, increased apoA-I/ABCA1 complex formation was concomitant with increased cellular phospholipid and cholesterol efflux (Fig. 1*C*). Several groups have reported binding studies with apoA-I conducted at 4 °C and the results have been somewhat inconsistent (14, 29). Here we demonstrate that the apoA-I cell association showed remarkable temperature dependence, suggesting that apoA-I binding to ABCA1 may be controlled by an energy-dependent process or, alternatively, the change in temperature may alter the lipid conformation in the plasma membrane, which then could affect apoA-I cell association. This result underscores the importance of using physiological temperatures to study apoA-I/ABCA1 interactions.

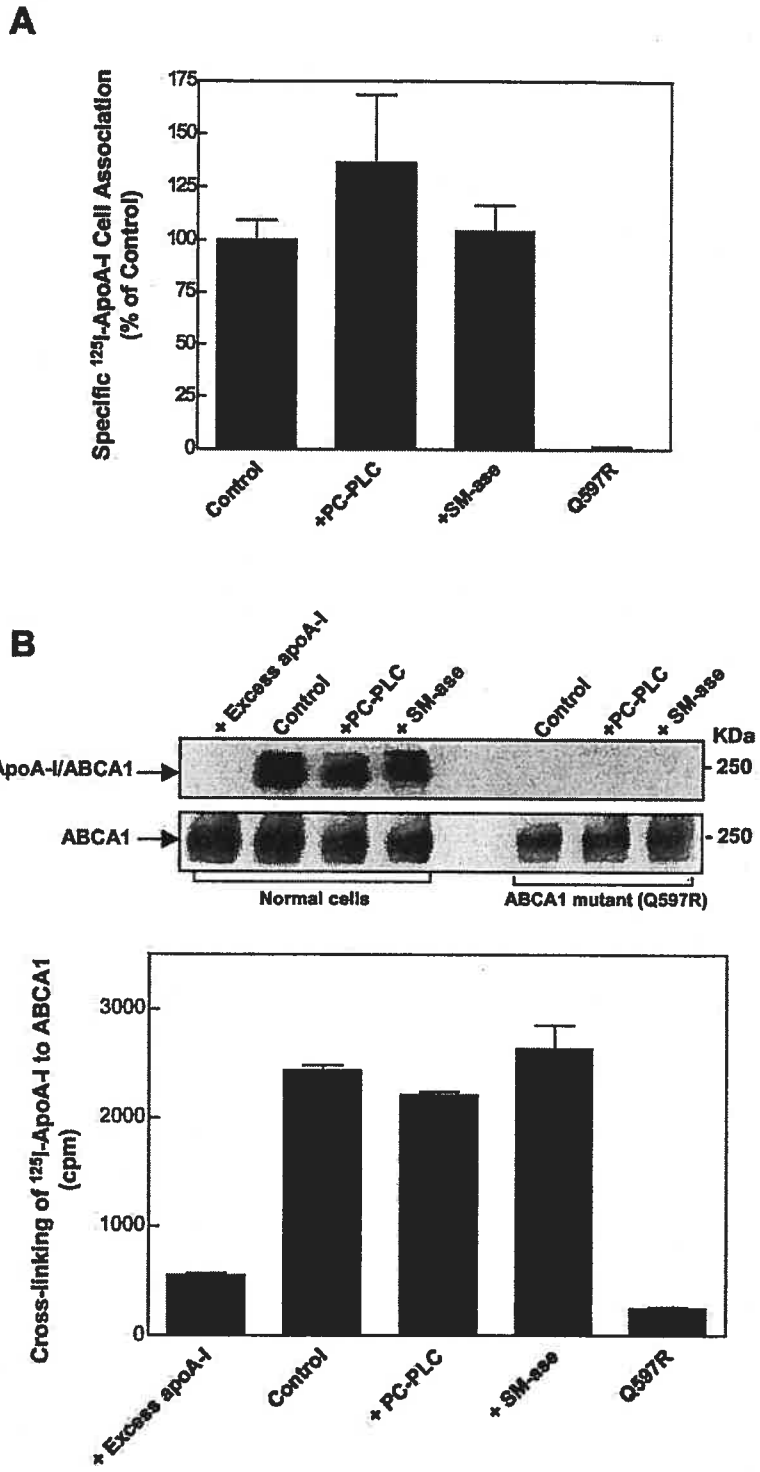


FIG. 3. **Effect of phospholipase treatment on apoA-I/ABCA1 interactions.** *A*, stimulated cells were incubated for 60 min at 37 °C in the presence or absence of 5 units/ml PC-PLC or 0.4 units/ml SM-ase. Cells were then incubated with 10 μg/ml of ^{125}I-apoA-I for 2 h at 37 °C. Specific ^{125}I-apoA-I binding was determined as described in Fig. 1*A*. Control value (100%) represent 24 ng of apoA-I/mg cell protein. ABCA1 mutant (Q597R) was used as a negative control. *B*, *upper panel*, intact stimulated normal or Q597R cells in 100-mm diameter dishes were incubated or not with PC-PLC or SM-ase as described above and then incubated with 10 μg/ml of ^{125}I-apoA-I for 1 h at 37 °C in the presence or absence of a 20-fold excess of unlabeled apoA-I (200 μg/ml). Cross-linking with DSP was performed as described above. Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 μg of total protein) were incubated with 10 μl of affinity-purified human anti-ABCA1 antibody for 20 h at 4 °C, followed by addition of protein A bound to Sepharose (30 μl). Immunoprecipitated samples were separated on 4–22.5% SDS-polyacrylamide gel electrophoresis and ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography. The ABCA1 protein was detected on the same membrane by anti-ABCA1 antibody. Lower panel, intact normal cells were incubated or not with PC-PLC or SM-ase as described above and then incubated with 10 μg/ml of ^{125}I-apoA-I for 1 h at 37 °C in the presence or absence of a 20-fold excess of unlabeled apoA-I. Quantitative cross-linking of ^{125}I-apoA-I to ABCA1 was performed as described under "Experimental Procedures." The amount of bound iodinated apoA-I to ABCA1 in the immunoprecipitates was determined by γ-counting. Results shown are representative of two different independent experiments.

To gain further insight into the relationships between the conformation/organization of apoA-I within lipidated HDL particles and its interaction with ABCA1, we performed competition assays that clearly showed that plasma pre-β_1-LpA-I, reconstituted HDL particles r(LpA-I), and native HDL_3 particles are poor competitors for the binding of ^{125}I-apoA-I to ABCA1 compared with lipid-free apoA-I (Fig. 2*A*). This experiment indicates an important role for the association of apoA-I with lipids in controlling apoA-I/ABCA1 interactions. Surprisingly, pre-β_1-LpA-I, which comprises apoA-I combined with only a small amount of phospholipids (30) had a 4-fold lower efficiency to interact with ABCA1 relative to lipid-free apoA-I (Fig. 2*A*). Previous studies established that the lipid composition of pre-β_1-LpA-I species as well as the conformation of apoA-I within these particles differ from those of spherical HDL (25, 30). Furthermore, pre-β_1-LpA-I is proposed to be an initial acceptor of cell-derived cholesterol (30). This supports the idea that pre-β_1-LpA-I removes cellular lipid by an aqueous diffusion process rather than an ABCA1-dependent pathway. The physiological relevance of the ABCA1-HDL interaction remains to be determined.

Although evidence have been presented demonstrating molecular interactions between ABCA1 and apoA-I (13, 14, 16), it remains controversial whether there is a "molecule-to-molecule contact" between apoA-I and ABCA1. Several competing models have been proposed for this interaction: 1) Burgess *et al.*

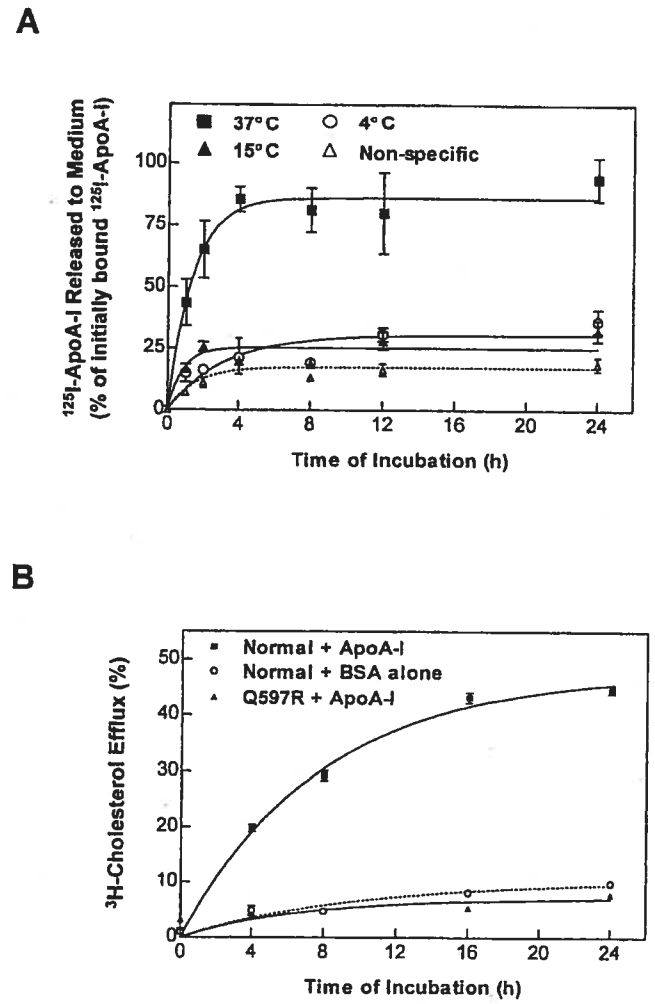


FIG. 4. **Dissociation of ^{125}I-apoA-I from stimulated fibroblasts and the kinetics of ABCA1-dependent cholesterol efflux.** *A*, stimulated cells in 24-well plates were incubated with 10 μg/ml of ^{125}I-apoA-I for 2 h at 37 °C. Nonspecific binding was determined in the presence of a 30-fold excess of unlabeled apoA-I and shown as the nonspecific. After washing to remove unbound ^{125}I-apoA-I, 0.5 ml of DMEM was added, and the plates were immediately incubated at either 37, 15, or 4 °C. At various time points, the radioactivity appearing in the medium was determined. Values represent the mean ± S.D. from triplicate wells. The initial binding value measured at t = 0 h was 0.24 ± 0.08 ng of apoA-I/μg cell protein. Similar results were obtained from two other control fibroblast cell lines. *B*, stimulated normal or Q597R cells were radiolabeled with [^{3}H]cholesterol and incubated with 10 μg/ml of apoA-I or 1 mg/ml of BSA at 37 °C for the indicated time points. Cholesterol efflux was determined as described under "Experimental Procedures." Values represent the mean ± S.D. from triplicate wells. Results shown are representative of four different independent experiments.

(31) suggested that phospholipids contained in the extracellular matrix of macrophages act as an initial tether point for apoA-I, bringing it into close proximity to membrane-bound ABCA1; 2) Chambenoit *et al.* (14) have reported that even though ABCA1 expression increases the amount of membrane bound apoA-I, its association with cellular membranes exhibits diffusional properties that are consistent with apoA-I binding to membrane lipids rather than an integral membrane protein. In the present study, experiments have been designed to answer this controversy and evidence was in fact obtained demonstrating that both of these models cannot be applied to apoA-I/ABCA1 interactions. Here, we demonstrate that treatment of intact stimulated cells with phospholipases (PC-PLC or SM-ase) affected neither the specific binding of ^{125}I-apoA-I nor apoA-I/ABCA1 cross-linking (Fig. 3, *A* and *B*). It is likely that apoA-I/ABCA1 interactions are due to a direct protein-protein contact, which is not dependent on the presence of membrane phosphatidylcholine or sphingomyelin. However, it is not excluded that other membrane phospholipids or the phospholipase lipid products may serve to bind the amphiphathic helix of apoA-I to ABCA1. This is consistent with a previous study by Smith *et al.* (32) showing that although ABCA1 expression is associated with an increase in cell surface phosphatidylserine level, the cellular association of apoA-I is not competed by annexin V, a phosphatidylserine binding protein. Moreover, Mendez *et al.* (33) documented that cholesterol and sphingomyelin-rich membrane rafts do not provide lipid for efflux promoted by apolipoproteins through the ABCA1-mediated lipid secretory pathway.

It has been suggested that the correct conformation of ABCA1 thought to be maintained by the ATP hydrolysis action of ABCA1 or its lipid flipping activity (14, 19) was necessary for apoA-I binding. Furthermore, recent studies from our laboratory and others have shown that ABCA1 phosphorylation by cAMP/PKA-dependent pathway plays an important role in the apoA-I lipidation reaction (20, 34, 35), suggesting that lipidation of apoA-I by ABCA1 is an active process. We confirmed and extended this observation by showing that ^{125}I-apoA-I dissociation from ABCA1 was almost completely inhibited at either 4 or 15 °C (Fig. 4*A*).

The structural requirements of apoA-I lipidation by ABCA1 have not yet been determined. However, in an attempt to understand this process in fibroblasts, we examined apoA-I lipidation reaction in a tissue culture model by monitoring the kinetic parameters of apoA-I dissociation from ABCA1. We initially hypothesized that any specific apoA-I dissociation from ABCA1 would be associated with a significant increase in apoA-I lipidation state, consistent with the concept that the transfer of phospholipid and cholesterol from the active site of ABCA1 transporter to apoA-I molecule weakens the interaction of apoA-I/ABCA1 and causes dissociation of the lipidated apoA-I product. Our hypothesis is supported by the finding that: 1) specific apoA-I dissociation from ABCA1 is rapid (Fig. 4*A*); 2) the association of apoA-I with lipids reduces its ability to interact with ABCA1; 3) the lipid translocase activity of ABCA1 generates α-LpA-I-like particles; and 4) ABCA1 did not mediate hydrolysis of apoA-I in fibroblasts. However, chlorpromazine has been shown to block cAMP-mediated cholesterol efflux in macrophages (36), supporting the idea that ABCA1 may be involved in the endocytosis and resecretion of apoA-I in macrophages. More thorough investigations are required to establish definitively a possible role of ABCA1 in the endocytosis of apoA-I in fibroblasts and macrophages.

Of interest, comparison of the dissociation rate constant of apoA-I from ABCA1 and apoA-I-mediated cholesterol efflux showed for the first time that apoA-I dissociation from ABCA1 is rapid ($t_{1/2}$ = 1.4 h, Fig. 4*A*). In contrast, in our stimulated cell culture system, apoA-I-mediated cholesterol efflux reached saturation after a 16-h incubation (Fig. 4*B*). Previous studies have demonstrated that lipid-free apolipoproteins access both cellular FC and PL during incubations of 4–24 h (37, 38), however, others such as Gillotte *et al.* (39) do show saturation in a short time frame. It should be noted that in their study the fibroblasts were not enriched with cholesterol, ABCA1 was not induced and the cells were labeled with a very high amount of [^{3}H]cholesterol and [^{3}H]choline. Our results suggest that each ABCA1 molecule at the cell surface may have multiple lipidation cycles, which may result in the lipidation of many apoA-I molecules by the same ABCA1 molecule. This concept is supported by an elegant study by Tall and coworkers (34) demon-

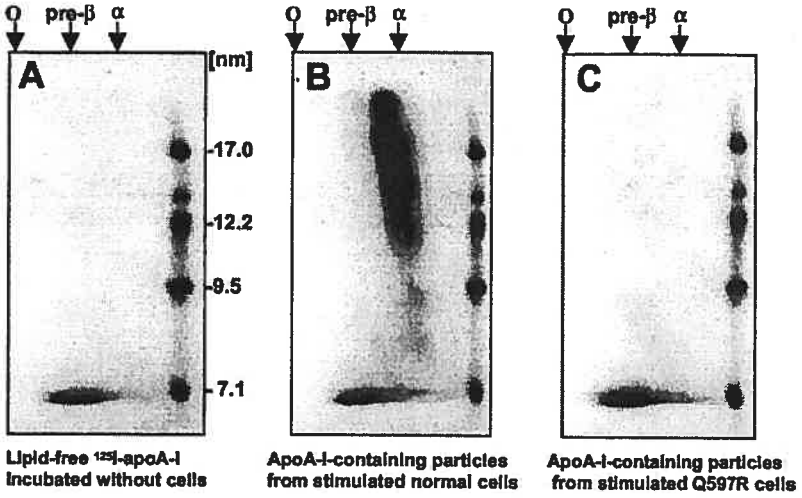


FIG. 5. **Analysis of lipid-free apoA-I charge and molecular diameter after incubation with either stimulated normal or ABCA1 mutant cells.** ^{125}I-apoA-I was incubated in DMEM/BSA for 24 h at 37 °C without cells (*A*) or with both stimulated normal and Q597R cells (*B* and *C*, respectively) for 24 h at 37 °C. Samples were separated by two-dimensional-PAGGE and ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film. Molecular size markers are indicated on the *right side* of each gel.

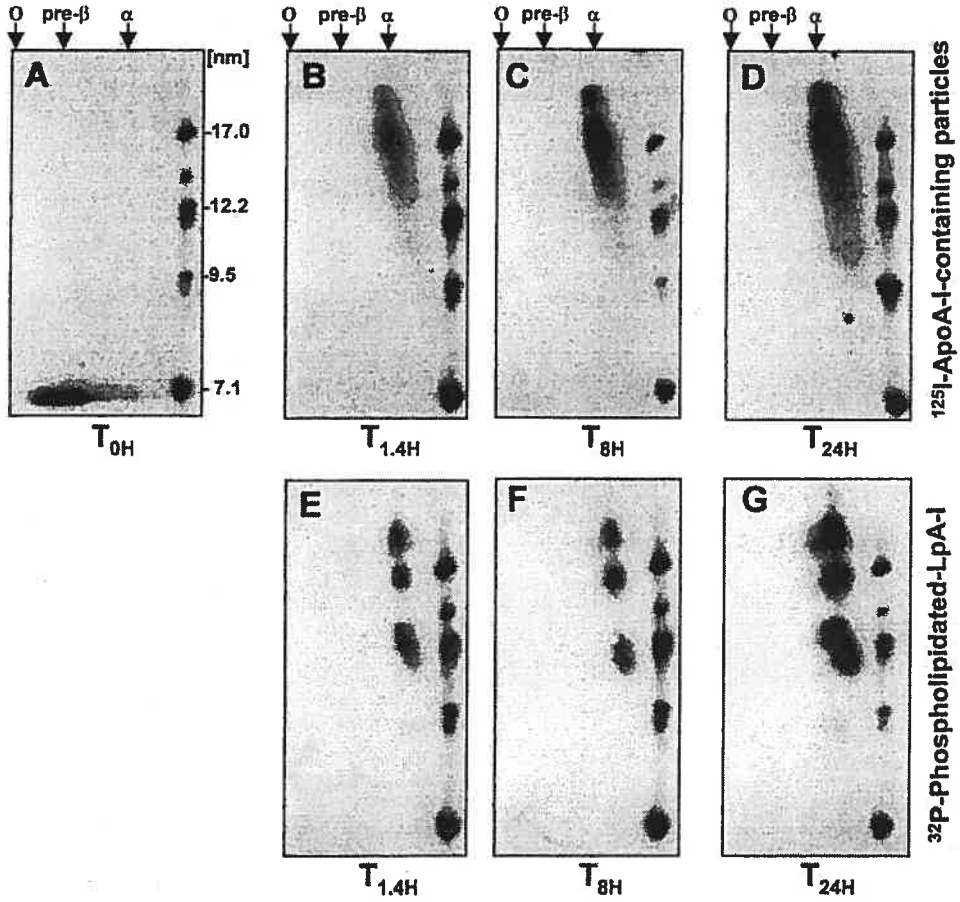


FIG. 6. **Time course of the formation of apoA-I-containing particles during the dissociation period.** *Upper panels*, stimulated normal cells were incubated with 10 μg/ml ^{125}I-apoA-I for 2 h at 37 °C. After washing to remove unbound ^{125}I-apoA-I, 15 ml DMEM was added, and the plate was immediately incubated at 37 °C for either 1.4, 8, or 24 h. The medium was recovered, concentrated and ^{125}I-apoA-I-containing particles at 1.4 h (*B*), 8 h (*C*), 24 h (*D*), or ^{125}I-apoA-I incubated in DMEM/BSA for 24 h at 37 °C without cells (*A*) were separated by two-dimensional-PAGGE. ^{125}I-apoA-I was directly detected by autoradiography using XAR-2 Kodak film. *Lower panels*, [^{32}P]orthophosphate-labeled normal cells were stimulated, and then incubated with 10 μg/ml unlabeled apoA-I for 2 h at 37 °C. After washing to remove unbound apoA-I, 15 ml DMEM was added, and the plate was immediately incubated at 37 °C for either 1.4, 8, or 24 h. The medium was recovered, concentrated, dialyzed, and ^{32}P-labeled phospholipids associated with apoA-I-containing particles were analyzed by two-dimensional-PAGGE. ^{32}P-labeled phospholipids associated with apoA-I-containing particles at 1.4 h (*E*), 8 h (*F*), or 24 h (*G*) was directly detected by autoradiography using XAR-2 Kodak film. Molecular size markers are indicated on the *right side* of each gel. Results shown are representative of two different independent experiments.

strating that apoA-I/ABCA1 interactions result in the dephosphorylation of the ABCA1 PEST sequence and thereby inhibits calpain degradation leading to an increase of both ABCA1 cell surface expression and activity.

Several laboratories have demonstrated that apoA-I incubated with cells including fibroblasts (37), CHO cells (40), and macrophages (38) was able to recruit phospholipid and cholesterol from the cells to form protein-lipid complexes. Our experiment presented in Fig. 5 shows that the apoA-I-lipid complexes thus formed during apoA-I incubation with stimulated normal cells represent a spectrum of particles with distinct molecular diameters. In contrast, lipid-free apoA-I was unable

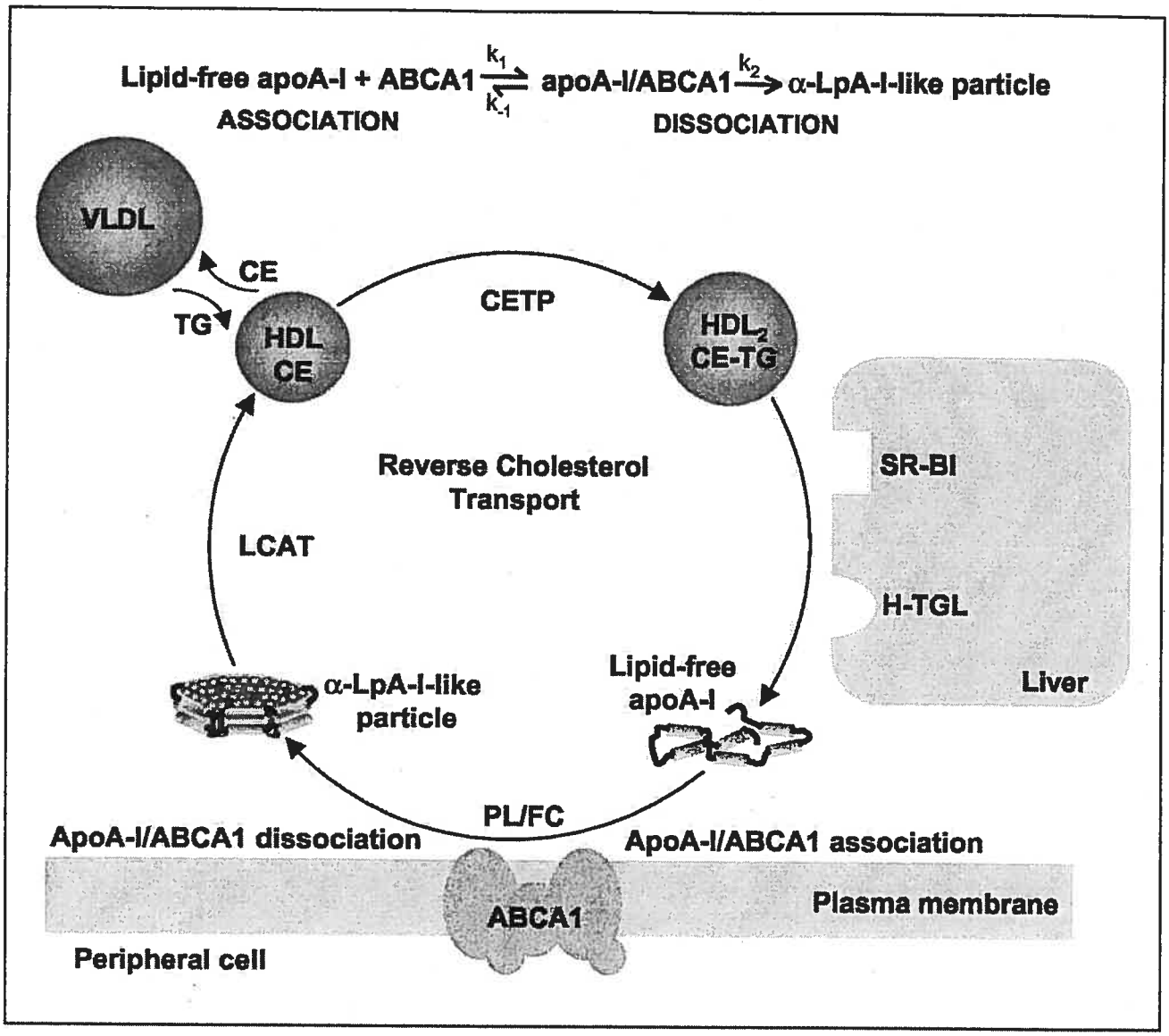


FIG. 7. **A proposed model of free apoA-I lipidation by ABCA1 in peripheral cells.** Cholesteryl ester-rich HDL_2 gain triacylglycerols from VLDL under the action of CETP. HDL_2 undergo lipolysis by the hepatic lipase (H-TGL) and a possible involvement of the SR-BI receptor generating lipid-free apoA-I, which can be rapidly lipidated by ABCA1 and form α-LpA-I-like particles. Continuous action of LCAT contributes to the maturation of α-LpA-I-like particles to form cholesteryl ester-enriched HDL. A model of apoA-I lipidation by ABCA1 can be proposed assuming that 1) initial binding of apoA-I to ABCA1 is irreversible or slowly reversible ($k_1 \gg k_{-1}$); 2) lipidated apoA-I (α-LpA-I-like particles) dissociate rapidly (k_2) from ABCA1 without any detectable reassociation; and 3) this system contains no other apolipoprotein that could compete for the binding of lipid-free apoA-I to ABCA1. *PL*, phospholipids; *FC*, free cholesterol; *TG*, triglycerides; *CE*, cholesteryl esters.

to form larger particles during its incubation with Q597R mutant cells. The interrelationship between these particles was unclear; however, a time course analysis of apoA-I-containing particles dissociated from ABCA1 (Fig. 6) showed nascent apoA-I-phospholipid complexes that exhibited α-electrophoretic mobility with a particle size ranging from 9 to 20 nm (designated α-LpA-I-like particles). The stability of the charge, molecular diameter and phospholipid species content of these nascent particles over a 24-h dissociation period did not support the existence of a clear precursor-product relationship between the various particles and provide strong support for their common origin. It is important to note that the newly formed α-LpA-I-like particles had distinctly different sizes, suggesting that larger particles contained both phospholipids and cholesterol whereas the smallest particles contained only phospholipids and apoA-I (41). Because of the absence of cholesterol acyltransferase activity in the extracellular medium to convert FC to cholesteryl ester, it is most likely that α-LpA-I-like particles are discoidal. Indeed, it was documented that a lipoprotein with a high concentration of phosphatidylinositol could have a high negative charge and consequently an α-electrophoretic mobility (42), consistent with our finding that α-LpA-I-like particles have a high content in phosphatidylinositol (14 ± 0.2%).

During the preparation of this article a study by Liu *et al.* (43) reported that incubation of apoA-I with macrophages leads to the formation of more than one type of lipidated apoA-I-containing particles with a molecular diameter of 6–16 nm. In addition, this study supports the idea that there is a simultaneous release of PL and FC to apoA-I molecules through a membrane microsolubilization process. It is interesting to contrast our results with those reported in that study, which demonstrated that apoA-I-containing particles have a smaller size and an important amount of apoA-I remaining in its lipid-free form. It is possible that the cell species used in the two studies affect ABCA1-dependent lipidation of apoA-I: we used

human fibroblasts and Liu *et al.* (43) used J774 macrophages. In addition our cells were loaded directly with FC (20 μg/ml) and were stimulated with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid and their cells were loaded with 25 μg/ml acetyl-LDL and induced with cAMP. More importantly, we observed that both the charge and diameter of the newly formed LpA-I-like particles are markedly different from those of lipid-free apoA-I (Figs. 5 and 6). We therefore suggest that our experimental design based on the analysis of LpA-I particles released during the dissociation period might be critical for the study of LpA-I product generated by a specific lipid translocase activity of ABCA1.

Evidence has been presented here demonstrating that only lipid-free apoA-I is able to interact efficiently with ABCA1 *in vitro* (Fig. 2). However, it seemingly paradoxical that lipid free-apoA-I molecules, which are not normally present in significant quantities in plasma (44), play a similar role *in vivo*. We postulate that lipid-free apoA-I generated during apoA-I-containing particles remodeling cycle (45) are rapidly lipidated by ABCA1 and form α-LpA-I-like particles or, alternatively, may be incorporated in preexisting plasma HDL. Our current results support the first hypothesis. We demonstrate that 50% of specifically bound ^{125}I-apoA-I was rapidly dissociated from ABCA1 at physiological temperatures ($t_{1/2}$ ~1.4 h) (Fig. 4*A*). At the same time, the majority of apoA-I-containing particles generated during the dissociation period was shown to be associated with phospholipids having an α-electrophoretic mobility (Fig. 6*E*). This concept is supported by recent study by Kee *et al.* (46) demonstrating that the electrophoretic mobility of ^{125}I-apoA-I (lipid-free) changed from pre-β to α- electrophoretic mobility only 2 min after injection into wild-type rabbits. In addition, the same study documented that hepatic lipase has the capacity to decrease the size of α-migrating HDL, in agreement with the earlier work of Barrans *et al.* (45).

Different kinetic models can be proposed to explain the mechanism of apoA-I/ABCA1 interaction. Here, our cell culture system represents a relatively simple model. However, in peripheral tissues and interstitial fluid, many other lipid-free apolipoproteins (*e.g.* apoE, apoJ, apoA-IV) might compete with apoA-I for ABCA1 binding. Our results show that apoA-I binding to ABCA1 was found to occur in a time- and concentration-dependent manner (Fig. 1*A*). Thus, apoA-I/ABCA1 association can be described as a receptor-ligand interaction or a protein-protein interaction in solution under apparent equilibrium condition (Fig. 7). On the other hand, the lipid translocase activity of ABCA1 transforms lipid-free apoA-I to α-LpA-I-like particle; here ABCA1 seems to act as an enzyme catalyzing the lipidation of the substrate. Although admittedly speculative, we believe that our data support this hybrid model better than either ligand/receptor or substrate/enzyme model. The important finding that the interaction of lipid-free apoA-I with ABCA1 generates only α-LpA-I-like particles might help to explain why lipid-free apoA-I is found in trace amounts in human plasma. Indeed, following the release of lipid-free apoA-I by the action of hepatic lipase on HDL_2 and a possible involvement of SR-BI in this process (47), lipid-free apoA-I molecules might be very rapidly lipidated by ABCA1 and transformed into α-LpA-I-like particles (Fig. 7).

The results presented in this study provide a biochemical basis for a cellular apoA-I lipidation pathway that involves ABCA1 protein in peripheral cells. This process plays *in vivo* a key functional role in the biogenesis of nascent HDL particles.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. ??, Issue of ???? ??, pp. 1–xxx, 2004
Printed in U.S.A.

Characterization of Oligomeric Human ATP Binding Cassette Transporter A1

POTENTIAL IMPLICATIONS FOR DETERMINING THE STRUCTURE OF NASCENT HIGH DENSITY LIPOPROTEIN PARTICLES*

Received for publication, June 21, 2004, and in revised form, July 23, 2004
Published, JBC Papers in Press, July 26, 2004, DOI 10.1074/jbc.M406881200

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The oligomeric structure of ABCA1 transporter and its function related to the biogenesis of nascent apoA-I-containing particles (LpA-I) were investigated. Using *n*-dodecylmaltoside and perfluoro-octanoic acid combined with non-denaturing gel, the majority of ABCA1 was found as a tetramer in ABCA1-induced human fibroblasts. Furthermore, using chemical cross-linking and SDS-PAGE, ABCA1 dimers but not the tetramers were found covalently linked. Oligomeric ABCA1 was present in isolated plasma membranes as well as in intracellular compartments. Interestingly, apoA-I was found to be associated with both dimeric and tetrameric, but not monomeric, forms of ABCA1. Neither apoA-I nor lipid molecules did affect ABCA1 oligomerization. Immunoprecipitation analysis showed that oligomeric ABCA1 did not contain other associated proteins. We next investigated the relationship between the oligomeric ABCA1 complex and the structure of LpA-I. Lipid-free apoA-I incubated with normal cells generated LpA-I with diameters between 9.5 and 20 nm. Subsequent isolation of LpA-I followed by cross-linking revealed the presence of four and eight apoA-I molecules per particle, whereas apoA-I incubated with ABCA1 mutant (Q597R) cells was unable to form such particles and remained in the monomeric form. These results demonstrate that: 1) ABCA1 exists as an oligomeric complex; and 2) ABCA1 oligomerization was independent of apoA-I binding and lipid molecules. The findings that the majority of ABCA1 exists as a tetramer that binds apoA-I, together with the observation that LpA-I contains at least four molecules of apoA-I per particle, support the concept that the homotetrameric ABCA1 complex constitutes the minimum functional unit required for the biogenesis of high density lipoprotein particles.

ABCA1 [1] is a 240-kDa protein belonging to a large family of conserved transmembrane proteins that transport a wide variety of substrates, including lipids, ions, amino acids, peptides, sugars, vitamins, steroid hormones, and drugs across cell membranes (1). ABC transporters have been associated with many diseases such as drug-resistant cancer (2), diabetes (3), and cystic fibrosis (4).

Apolipoprotein (apo) A-I binding to the extracellular domain of ABCA1 results in the activation of apoA-I lipidation, a key step in the reverse cholesterol transport process, one of the major mechanisms by which high density lipoprotein (HDL) may protect against atherosclerotic vascular disease (5–7). The molecular interaction of apoA-I with ABCA1 promotes cholesterol and phospholipid efflux from peripheral cells and macrophages. However, Brewer and colleagues (8) recently reported that hepatic ABCA1 is a key protein for the formation and maintenance of plasma HDL levels. Moreover, the importance of ABCA1 in the lipidation of apoA-I is highlighted by the finding that over 50 mutations in the ABCA1 gene have been associated with a variety of clinically distinct HDL deficiency diseases including Tangier disease and familial HDL deficiency (9–11). These patients are characterized by excess cholesterol accumulation in macrophages, low plasma HDL levels, and increased risk of coronary artery atherosclerosis (12).

ABC transporters typically consist of two multispanning membrane domains that serve as a pathway for the translocation of substrates across membranes and two ATP binding cassettes or nucleotide binding domains that provide the energy for substrate transport (13, 14). These domains are found either on a single long polypeptide chain, as in the case of cystic fibrosis transmembrane conductance regulator and the multidrug resistance proteins, P-glycoprotein and MRP1, or as a complex of two identical or similar "half-molecule" subunits each having an multispanning membrane domain and an nucleotide binding domain, as found in the TAP1/TAP2 ABC transporters associated with peptide antigen processing. ABCA1 belongs to the first category because it consists of a single polypeptide composed of two arranged halves. Each half contains an multispanning membrane domain followed by a cytoplasmic nucleotide binding domain. A distinguishing feature of ABCA1 is the presence of a large exocytoplasmic domain that connects the first transmembrane segment to the multispanning membrane domain in each half of the protein (15).

Although the ABCA1 molecule is well characterized, very

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[1] The abbreviations used are: ABCA1, ATP binding cassette AI; PAGGE, polyacrylamide non-denaturing gradient gel electrophoresis; apo, apolipoprotein; DSP, dithiobis(succinimidylpropionate); DTT, dithiothreitol; HDL, high density lipoprotein; LpA-I, nascent apoA-I-containing particle; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MWCO, molecular weight cut-off.

little is known concerning its quaternary structure and its functional properties related to the formation of nascent apoA-I-containing particles. To date, no studies have directly assessed the multimeric structure of human ABCA1. It was therefore the aim of the present study to provide evidence for the existence of oligomeric ABCA1 complex, to demonstrate how ABCA1 forms could be affected by apoA-I or lipid molecules, and to examine the impact of the oligomeric ABCA1 complex on the structure of nascent apoA-I-containing particles in a cell culture model.

EXPERIMENTAL PROCEDURES

Patient Selection—For the present study, we selected fibroblasts from three normal control subjects and one patient with Tangier disease (homozygous for Q597R at the ABCA1 gene). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Centre. Separate consent forms for blood sampling, DNA isolation, and skin biopsy were provided.

Cell Culture—Human skin fibroblasts were obtained from 3.0-mm punch biopsies of the forearm of patients and healthy control subjects and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% non-essential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Human green fluorescent protein (GFP)-ABCA1 expressing Chinese hamster ovary cells were generously provided by Dr. Sean Davidson, Department of Pathology and Laboratory Medicine, University of Cincinnati, and were characterized and cultured as described previously (16).

Human Plasma apoA-I and apoE3—Purified plasma apoA-I (Biodesign) was resolubilized in 4 M guanidine-HCl and dialyzed extensively against PBS buffer and used within 24 h. ApoA-I was iodinated with ^{125}I-labeled iodine by IODO-GEN® (Pierce) to a specific activity of 800–2500 cpm/ng of apoA-I. Purified human plasma apoE3 was a gift from Dr. Karl H. Weisgraber (Gladstone Institutes of Cardiovascular Disease, San Francisco, CA).

Solubilization of Cell Proteins by n-Dodecylmaltoside and Perfluoro-octanoic Acid—Normal fibroblasts in 100-mm diameter dishes were stimulated with 2.5 μg/ml 22-(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Cells were then lysed at 4 °C with PBS containing 0.5% *n*-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble materials. In separate experiments, cells were lysed with 0.8% perfluoro-octanoic acid as described by Ramjeesingh *et al.* (17). After solubilization of cell proteins and centrifugation at 11,000 × *g*, 4 °C, for 10 min, the supernatants were treated or not with 50 mM dithiothreitol (DTT) for 30 min at 37 °C, and then the samples were separated by non-denaturing gradient gel electrophoresis (3–15%) as described previously (18).

Chemical Cross-linking and Immunoprecipitation Analysis—Chemical cross-linking was performed as described by Tall and colleagues (19) with a minor modification. Fibroblasts were grown to confluence in 100-mm diameter dishes and then stimulated or not with 2.5 μg/ml 22-(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h in DMEM/bovine serum albumin. Cells were incubated in the presence or absence of 10 μg/ml ^{125}I-apoA-I in DMEM/bovine serum albumin for 2 h at 37 °C. Cells were then placed on ice for 15 min and washed three times with PBS. Dithiobis(succinimidylpropionate) (DSP, cross-linker agent) was dissolved immediately before use in Me_2SO and diluted to 500 μM in PBS. Six ml of DSP solution was added in each well. Cells were then incubated at room temperature for 30 min; the medium was removed, and the cells were washed twice with PBS. Cells were lysed at 4 °C with 20 mM Tris, 5 mM EDTA, 5 mM EGTA; pH 7.5) containing 0.5% *n*-dodecylmaltoside, and the suspension was allowed to stand for 10 min at 4 °C in the presence of a protease inhibitor mixture (Roche Diagnostics). Either apoA-I/ABCA1 complex or ABCA1 alone was immunoprecipitated with an affinity-purified polyclonal anti-ABCA1 antibody (Novus Biologicals) as described previously (20, 21). After SDS-gel electrophoresis, ABCA1 was detected by an affinity-purified human anti-ABCA1 antibody. The presence of labeled ^{125}I-apoA-I/ABCA1 complexes was directly detected by autoradiography by using XAR-2 Kodak film.

Isolation of Nascent LpA-I Particles and Cross-linking with DSP—^{125}I-apoA-I (10 μg/ml) was incubated in DMEM for 24 h at 37 °C with either stimulated normal or Q597R cells. LpA-I particles were isolated by using ultrafiltration (spiral ultrafiltration cartridge, MWCO 100,000, Amicon) to discard any lipid-free apoA-I. LpA-I particles were further dialyzed by using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I. Cross-linking of isolated LpA-I was performed as described by Davidson and Hilliard (22) with slight modifications. 15 μg of isolated LpA-I generated by normal cells, apoA-I incubated with Q597R cells, or lipid-free apoA-I incubated without cells was adjusted to 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, with a final protein concentration of 1 μg/μl. Immediately before an experiment, 1 mg of DSP was dissolved in 1000 μl of Me_2SO to a final concentration of 1 μg/μl. The dissolved DSP was added to the reaction mixture for 10 DSP to 1 apoA-I molar ratio on ice. The reaction was incubated at 4 °C for 24 h with periodic vortexing. The reaction was quenched by adding 1 M Tris, pH 7.8, to a final Tris concentration of 100 mM. To rule out the possibility that cross-linking conditions might affect the number of apoA-I molecules per particle, the molar ratio of DSP to apoA-I was varied from 5/1 to 20/1, and incubations were conducted at different temperatures (4 °C, 37 °C, and room temperature).

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously (23) with slight modifications. Confluent cells were stimulated and then cross-linked with DSP as described above. Surface proteins were biotinylated with 500 μg/ml *N*-hydroxysulfosuccinimydyl-S,S-biotin (Pierce) for 30 min at 4 °C. Cells were then washed with ice-cold quench buffer (1 M Tris-HCl (pH 7.5)) and twice with ice-cold PBS. The cells were lysed at 4 °C with 20 mM Tris, 5 mM EDTA, 5 mM EGTA (pH 7.5) containing 0.5% *n*-dodecylmaltoside in the presence of a protease inhibitor mixture and then homogenized with 40 strokes in a tight fitting Dounce homogenizer. After centrifugation at 1000 × *g*, 4 °C, for 10 min to remove unbroken cells and nuclei, 200 μg of protein from the supernatant was added to 30 μl of streptavidin-Sepharose (Amersham Biosciences) and incubated overnight on a platform mixer at 4 °C. The beads was pelleted and washed three times with lysis buffer. Cross-linking, SDS-PAGE, and detection of ABCA1 were performed as described above.

Metabolic Labeling and Immunoprecipitation of ABCA1—Metabolic labeling of ABCA1 was performed as described by Wang and Oram (24). Briefly, either stimulated or unstimulated normal cells were labeled with 150 μCi/ml [^{35}S]methionine for 4 h. Cells were then lysed at 4 °C with lysis buffer containing 0.5% *n*-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble materials. The supernatants were immunoprecipitated with an anti-ABCA1 antibody. Immunoprecipitated samples were separated on 4–22.5% SDS-PAGE, and ^{35}S-labeled ABCA1 was directly detected by autoradiography.

Separation of Lipoproteins by Two-dimensional Non-denaturing Gradient Gel Electrophoresis (PAGGE)—ApoA-I-containing particles were separated by two-dimensional-PAGGE, as described previously (18). Briefly, samples (30–100 μl) were separated in the first dimension (according to their charge) by 0.75% agarose gel electrophoresis (100 V, 3 h, 4 °C) and in the second dimension (according to the size) by 5–23% polyacrylamide concave gradient gel electrophoresis (125 V, 24 h, 4 °C). Iodinated high molecular weight protein mixture (7.1–17.0 nm, Amersham Biosciences) was run as a standard on each gel. Electrophoretically separated samples were electrotransferred (30 V, 24 h, 4 °C) onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences). ^{125}I-apoA-I was directly detected by autoradiography by using XAR-2 Kodak film.

RESULTS

In the present study, we have examined the multimeric status of human ABCA1 transporter in normal intact fibroblasts stimulated with 22-(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid (22OH/9CRA) by using both *n*-dodecylmaltoside and perfluoro-octanoic acid combined with non-denaturing gel electrophoresis. These detergents at appropriate concentrations do not break the non-covalent interactions between protein subunits of an oligomer allowing determination of the oligomeric structure of ABCA1 complex. As shown in Fig. 1*A*, detection of ABCA1 by anti-ABCA1 antibody, after separation of total cell lysate solubilized by a non-ionic detergent *n*-dodecylmaltoside (0.5%) on non-denaturing gel (3–15%), revealed both a major and minor bands. The major band migrated as an ~950-kDa complex, consistent with the molecular mass of tetramers. The minor band migrated as a larger complex, possibly an oligomer higher than tetramer, whereas the band with an apparent molecular mass of ~550 kDa is likely a dimer. On the other hand, using DTT as a reducing agent, we observed that all the oligomeric forms were reduced to the monomeric form with a

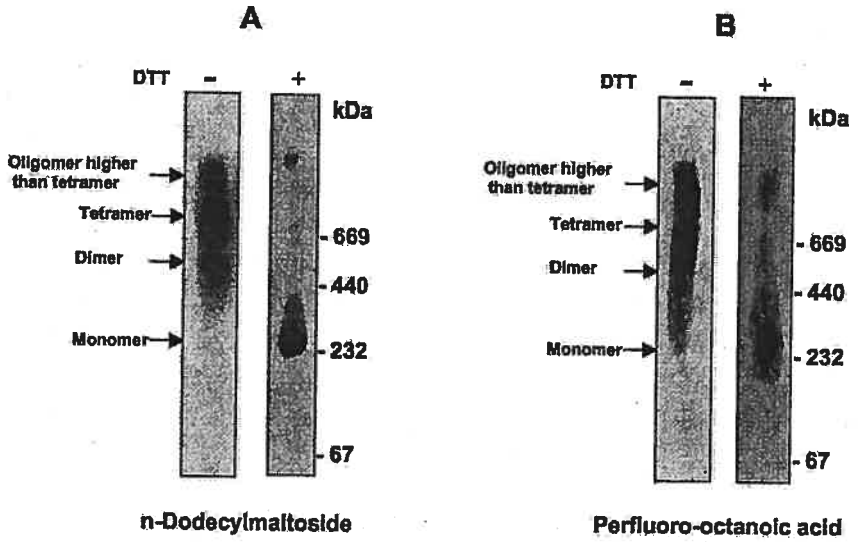


FIG. 1. **Analysis of oligomeric ABCA1 complex by PAGGE.** *A*, normal fibroblasts in 100-mm-diameter dishes were stimulated with 2.5 μg/ml 22-(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Cells were then lysed at 4 °C with PBS containing 0.5% *n*-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble material. The supernatants were treated or not with 50 mM DTT for 30 min at 37 °C and then separated by non-denaturing PAGE. After electrophoresis, ABCA1 was detected with an affinity-purified polyclonal anti-ABCA1 antibody. *B*, stimulated fibroblasts were lysed at 4 °C with PBS containing 0.8% perfluoro-octanoic acid. The supernatants were treated or not with 50 mM DTT for 30 min at 37 °C and then separated by PAGGE. ABCA1 complex was detected as described in *A*. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa) were used as markers.

molecular mass of ~250 kDa reported for complex glycosylated ABCA1 protein as estimated by using SDS-PAGE (21). Similar results were also observed with a mild ionic detergent perfluoro-octanoic acid (Fig. 1*B*), suggesting that the oligomeric ABCA1 observed was not due to the use of a specific detergent. Interestingly, we did not detect any ABCA1 with the size of a monomer on non-denaturing gel in the absence of DTT.

To determine further whether the oligomeric ABCA1 complex exists in living cells, chemical cross-linking was performed as described by Tall and colleagues (19). As shown in Fig. 2*A*, using SDS-PAGE under non-reducing conditions, ABCA1 migrated at either the monomeric or dimeric molecular masses (~250 and ~500 kDa, respectively), whereas the monomer was predominant in the presence of DTT, indicative of disulfide bond contribution in dimer formation. On the other hand, a chemical cross-linker, DSP, was applied to the surface of intact normal fibroblasts to assess the quaternary structure of ABCA1. We found that immunoreactive ABCA1 in cells treated with DSP migrated primarily as a monomer (~250 kDa), as dimers (~500 KDa), or as a larger complex with a mass greater than that predicted for either a monomer or a dimer (Fig. 2*B*). This larger band is likely a tetramer. DTT reduced all the oligomeric forms to the monomeric ABCA1. To assess further the subcellular distribution of oligomeric ABCA1, we employed surface biotinylation to isolate ABCA1 associated with plasma membrane. Cross-linking of intact normal stimulated fibroblasts followed by biotinylation and detection of ABCA1 on SDS-PAGE showed three bands associated with plasma membrane, corresponding to monomeric, dimeric, and tetrameric ABCA1 (Fig. 2*C*, *left panel*). The three ABCA1 forms were also detected in the intracellular compartments (Fig. 2*C*, *right panel*).

Having determined that oligomeric ABCA1 complex was present in normal human fibroblasts treated with 22OH/9CRA, the question was posed whether ABCA1 induction in fibroblasts may cause self-association events that are non-physiological. We next examined the presence of oligomeric ABCA1 complex in Chinese hamster ovary cells overexpressing human ABCA1. We found that the monomeric and dimeric ABCA1 forms were present on SDS-PAGE under non-reducing conditions, whereas tetrameric ABCA1 was detected in the presence of the cross-linker reagent (DSP) (data not shown). To verify that the oligomerization of ABCA1 is not due to oxidation during cell lysis and membrane preparation, 5,5-dithiobis-2-nitrobenzoic acid was used as an agent that inhibited disulfide bond formation and the dimerization (25). We found that the absence or presence of 5,5-dithiobis-2-nitrobenzoic acid did not prevent the dimerization of ABCA1.

Because the physical interactions between apoA-I and ABCA1 have been proposed to be important in the lipidation of apoA-I (26), the question was raised whether lipid-free apoA-I could bind to different ABCA1 forms. Stimulated cells were incubated or not with 10 μg/ml ^{125}I-apoA-I for 2 h at 37 °C, and then cross-linking with DSP was performed. As shown in Fig. 3*B*, ^{125}I-apoA-I associated with ABCA1 co-localized with both dimeric and tetrameric ABCA1 complex (Fig. 3*A*), whereas ^{125}I-apoA-I was not found associated with monomeric ABCA1 (Fig. 3). Moreover, the absence or presence of apoA-I did not affect ABCA1 oligomerization (Figs. 2*B* and 3*A*, respectively). To verify that ABCA1 oligomerization was not dependent on the presence of lipids, cell lysates were delipidated or not (three times) with ethanol-ether 3:1, and then cross-linking was performed. Removal of total cellular lipids did not prevent ABCA1 oligomerization (data not shown). To determine whether the oligomeric ABCA1 is a homo- or hetero-oligomer, either stimulated or unstimulated normal fibroblasts were labeled with [^{35}S]methionine, and then ^{35}S-labeled ABCA1 was immunoprecipitated with an anti-ABCA1 antibody. As shown in Fig. 4, the human anti-ABCA1 antibody specifically precipitated no other proteins except human ABCA1. Although it cannot be ruled out that other proteins co-migrate with ABCA1 on SDS-PAGE, the low amount of detectable ^{35}S-labeled material in unstimulated cells did not support this possibility.

To determine the relationship between oligomeric ABCA1 complex and the structural properties of nascent apoA-I-containing particles in our cell culture model, stimulated cells either from normal or from Tangier disease (Q597R) subjects in 100-mm diameter dishes were incubated with 10 μg/ml ^{125}I-apoA-I in 6 ml of DMEM for 24 h at 37 °C. The medium was concentrated by ultrafiltration (spiral ultrafiltration cartridge,

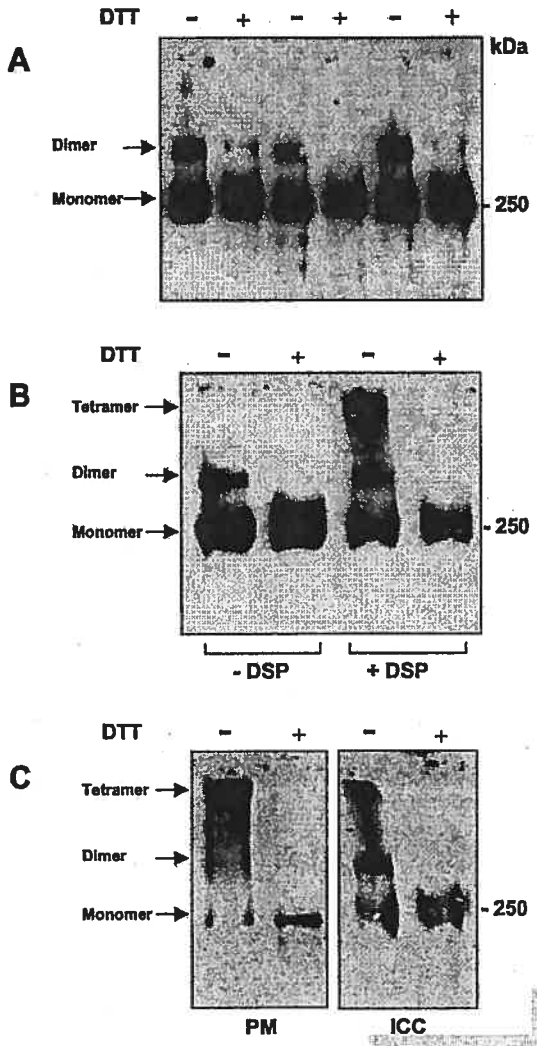


FIG. 2. **Chemical cross-linking of ABCA1 in intact fibroblasts and the cellular localization of the oligomeric ABCA1 complex.** *A*, normal fibroblasts in 100-mm diameter dishes were stimulated with 2.5 μg/ml 22-(*R*)-hydroxycholesterol and 10 μM 9-*cis*-retinoic acid for 20 h. Cells were then lysed at 4 °C with lysis buffer containing 0.5% *n*-dodecylmaltoside in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble material. The supernatants were treated or not with 50 mM DTT for 30 min at 37 °C and then separated by SDS-PAGE (4–22.5%) in triplicate. ABCA1 was detected as in Fig. 1. *B*, stimulated cells were cross-linked or not with 500 μM DSP, and the cells were lysed and reduced or not with DTT as described above for *A*. After electrophoresis on SDS-PAGE, ABCA1 was detected by an anti-ABCA1 antibody. *C*, stimulated cells were cross-linked with DSP, and surface biotinylation was employed to isolate ABCA1 associated with the plasma membrane (*PM*) as described under "Experimental Procedures." ABCA1 associated with intracellular compartments (*ICC*) was immunoprecipitated by an anti-ABCA1 antibody. Samples containing either plasma membrane or intracellular compartments were reduced or not with DTT and then separated by SDS-PAGE. ABCA1 was detected by an anti-ABCA1 antibody.

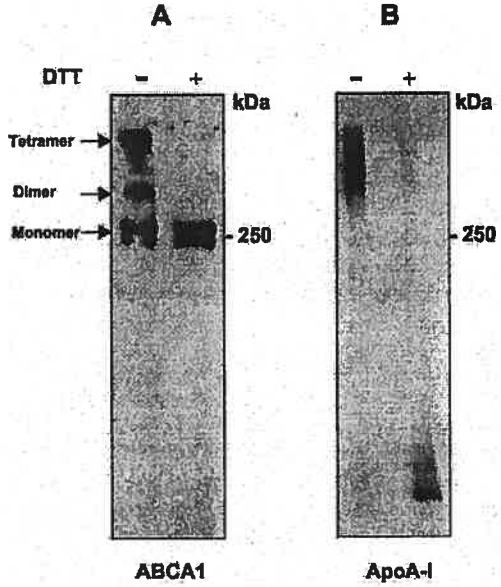


FIG. 3. **Association of apoA-I with the oligomeric ABCA1 complex.** *A*, stimulated normal cells were incubated with 10 μg/ml ^{125}I-apoA-I for 2 h at 37 °C. Cross-linking with DSP was performed as described above. Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 μg of total protein) were incubated with 10 μl of affinity-purified human anti-ABCA1 antibody for 20 h at 4 °C followed by the addition of protein A bound to Sepharose (30 μl). Immunoprecipitated samples were reduced or not with 50 mM DTT for 30 min at 37 °C and then separated on 4–22.5% SDS-PAGE. ^{125}I-apoA-I/ABCA1 complexes were directly detected by autoradiography. The ABCA1 protein was detected by an anti-ABCA1 antibody.

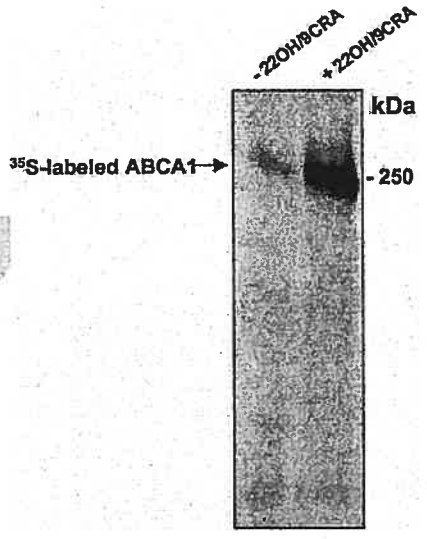
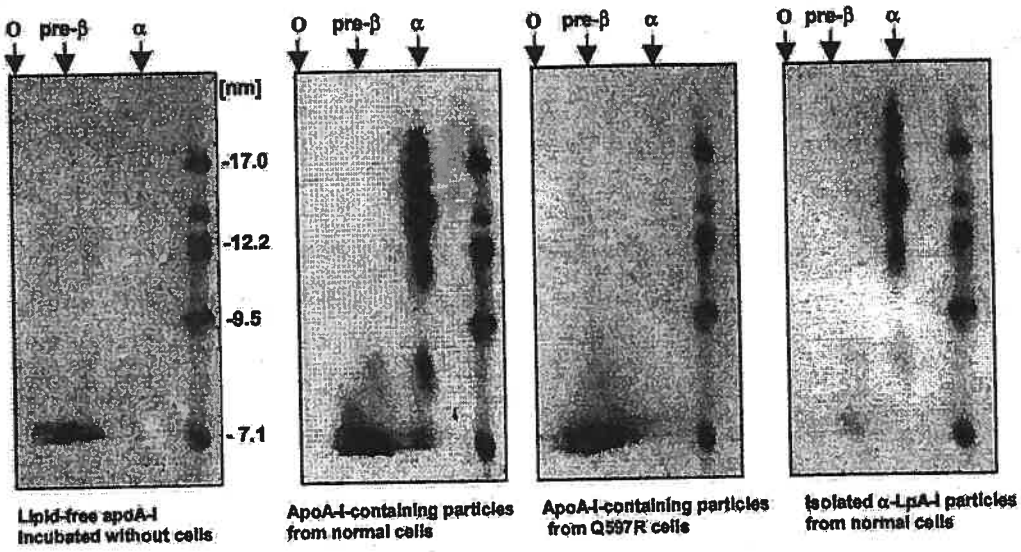


FIG. 4. **Immunoprecipitation of ^{35}S-labeled ABCA1.** Either stimulated or unstimulated normal cells were labeled with 150 μCi/ml [^{35}S]methionine for 6 h as described under "Experimental Procedures." Cells were then lysed at 4 °C with lysis buffer in the presence of a protease inhibitor mixture followed by low speed centrifugation to remove insoluble material. The supernatants were immunoprecipitated with an anti-ABCA1 antibody. Immunoprecipitated samples were separated on 4–22.5% SDS-PAGE, and ^{35}S-labeled ABCA1 was directly detected by autoradiography.

MWCO 10,000, Amicon), and ^{125}I-apoA-I-containing particles were electrophoretically separated by two-dimensional-PAGGE. As shown in Fig. 5, *upper panels*, apoA-I-containing particles generated by stimulated normal cells exhibited α-electrophoretic mobility with a particle diameter ranging from 9.5 to 20 nm (2nd *gel*). In contrast, lipid-free apoA-I incubated with stimulated ABCA1 mutant (Q597R) cells was unable to form such particles (*third gel*), which had a molecular diameter and charge similar to the lipid-free apoA-I incubated in the same conditions without cells (*first gel*). We next isolated LpA-I particles by using ultrafiltration (spiral ultrafiltration cartridge, MWCO 100,000, Amicon) to discard any lipid-free apoA-I. LpA-I particles were further dialyzed by using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I. As shown in the *fourth gel*, isolated LpA-I particles did not contain any significant amount of lipid-free apoA-I.

To further characterize the structure of LpA-I particles generated by ABCA1, isolated LpA-I particles formed by oligomeric ABCA1 complex were incubated with cross-linking reagent DSP, a homobifunctional cross-linker that interacts with the



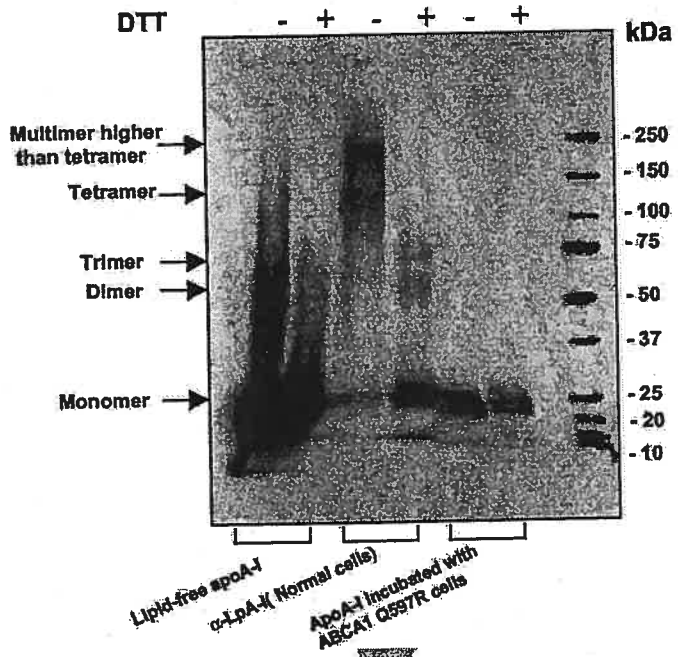


FIG. 5. **Characterization of nascent LpA-I particles generated by the oligomeric ABCA1 complex.** *Upper panels,* ^{125}I-apoA-I (10 μg/ml) was incubated in DMEM for 24 h at 37 °C without cells (*first gel*) or with both stimulated normal and Q597R cells (*second* and *third gels*, respectively) for 24 h at 37 °C. Samples were separated by two-dimensional-PAGGE. We next isolated LpA-I particles by using ultrafiltration (spiral ultrafiltration cartridge, MWCO 100,000, Amicon) to discard any lipid-free apoA-I. LpA-I particles were further dialyzed by using a dialysis membrane with a MWCO of 50,000 to remove any remaining lipid-free apoA-I (*4th gel*). ^{125}I-apoA-I was directly detected by autoradiography by using XAR-2 Kodak film. *Molecular size markers* are indicated on the *right side* of each gel. *Lower panel,* isolated LpA-I generated by normal cells, apoA-I incubated with Q597R cells, or lipid-free apoA-I incubated without cells were treated with DSP as described under "Experimental Procedures." The samples were reduced or not with 50 mM DTT for 60 min at 37 °C and then separated on 8–27% SDS-PAGE. ^{125}I-apoA-I was directly detected by autoradiography by using XAR-2 Kodak film. *Molecular size markers* are indicated on the *right side* of the gel. Results shown are representative of four different independent experiments.

ε-amine group on the side chain of lysine residues. The cross-linking can occur both intra- and intermolecularly but only between Lys residues within the reagent spacer arm length of 12 Å (27). This homobifunctional amine-specific cross-linker also possesses a cleavable disulfide bond. Either lipid-free apoA-I incubated without cells or lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells were used as controls. As shown in Fig. 5, *lower panel*, we consistently observed that LpA-I particles generated by stimulated normal cells contained either four or eight molecules of apoA-I per particle (~100 and 200 kDa, respectively), which could mostly be reduced back to a monomer by cleavage of the cross-link with DTT. In contrast, both lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells and lipid-free apoA-I incubated without cells remained in the monomeric form following cross-linking. To rule out the possibility that cross-linking conditions or iodination of apoA-I

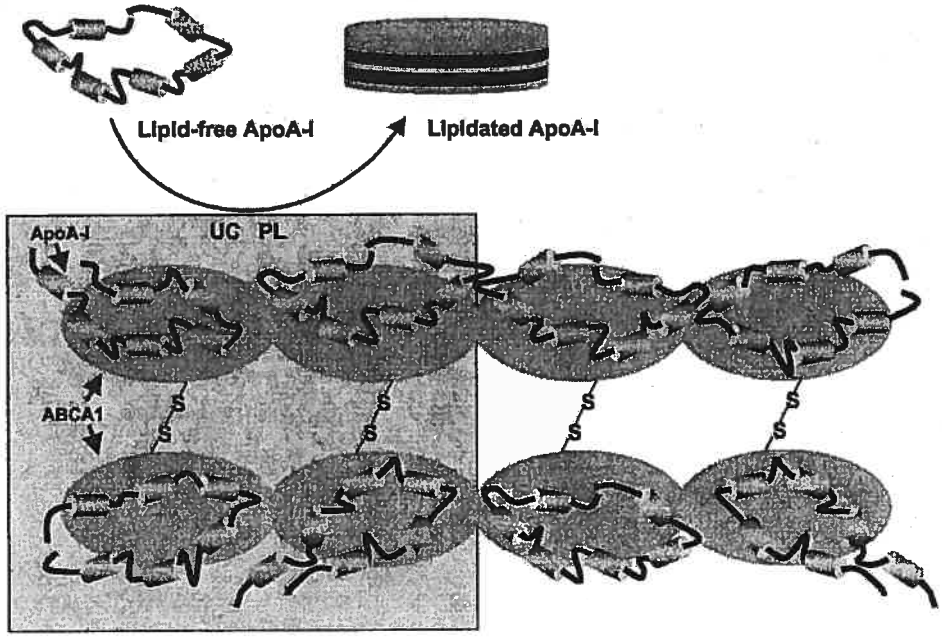


FIG. 6. **A proposed model of lipid-free apoA-I lipidation by the oligomeric ABCA1 complex in peripheral cells.** Different types of molecular interactions exist between ABCA1 molecules. It is likely that the sulfhydryl groups between the ABCA1 subunits form disulfide bonds. There is no evidence of intermolecular disulfide bonds that link either a homo-dimeric or the homo-tetrameric ABCA1 subunits together. The homo-tetrameric complex designated as the minimum functional unit binds four molecules of apoA-I at the same time followed by the transfer of phospholipids and cholesterol from the active site of the oligomeric ABCA1 complex, allowing the assembly of four molecules of apoA-I on the same particle (lipidated apoA-I). The transfer of lipids to apoA-I molecules may weaken the interaction of nascent LpA-I with the oligomeric ABCA1 complex and causes dissociation of the lipidated product. The organization of apoA-I molecules within nascent α-LpA-I is unknown. The present organization of apoA-I within LpA-I is a simple illustration of apoA-I lipidation by the oligomeric ABCA1 complex. *PL*, phospholipids; *UC*, unesterified cholesterol.

might affect the number of apoA-I molecules per particle, the molar ratio of DSP to apoA-I was varied from 5/1 to 20/1, and incubations were conducted at different temperatures (4 °C, 37 °C, and room temperature). On the other hand, unlabeled apoA-I was used in some experiments. We found that neither the amount of the cross-linker nor the iodination of apoA-I affected significantly the number of apoA-I molecules per particle (data not shown).

DISCUSSION

The lipid translocase activity of ABCA1 transporter has been implicated in important functions, including the regulation of intracellular lipid trafficking and the lipidation of lipid-poor apolipoproteins to form nascent HDL-particles (20, 28, 29). It is key that we understand the functional properties of this protein and structural basis for its activity. For the first time, we present evidence that a majority of human ABCA1 exists in intact fibroblasts as a homo-tetramer with a possible higher order of oligomerization (Fig. 1). Similar results were also observed with Chinese hamster ovary cells overexpressing human ABCA1, suggesting that the oligomeric ABCA1 complex observed was not due to the use of specific cell types. Interestingly, the absence of ABCA1 monomer as assessed by non-denaturing gel electrophoresis (Fig. 1) suggests that the oligomeric state could even be an essential prerequisite for its sorting, in the trans-Golgi-network and to secretory vesicles. This is consistent with previous studies demonstrating that other ABC transporters such as cystic fibrosis transmembrane conductance regulator, MRP1, or ABCG2 function as either dimers or tetramers (17, 30, 31).

Although the molecular mechanism of apoA-I binding to oligomeric ABCA1 has not been elucidated, the present study shows that lipid-free apoA-I binds to both dimeric and tetrameric ABCA1 complex (Fig. 3). We believe that these structures are physiologically relevant; it is likely that the tetrameric ABCA1 complex constitutes the minimum functional structure required for the apoA-I lipidation process. However, it is possible that the dimeric ABCA1 is a functional lipid transporter and that other oligomeric ABCA1 complexes function only as a regulator for the level of a dimeric form. Our observation that only a minor proportion of oligomeric ABCA1 exists as a dimer in living cells (Fig. 1*A*) did not support such a mechanism.

The proposed mechanism of tetrameric ABCA1 complex as the minimum functional unit required for the lipidation of apoA-I was further strengthened by our results demonstrating that nascent apoA-I-containing particles generated by the lipid translocase activity of ABCA1 contain either four or eight molecules of apoA-I per particle. Thus, we provide further evidence for a functional link between oligomeric ABCA1 transporter and the multimeric structure of nascent apoA-I-containing particles. We postulate that functional oligomeric ABCA1 complex is required for the lipid transfer and the assembly of multiple molecules of apoA-I on the same particle. Our current results support this hypothesis. We demonstrate that lipid-free apoA-I incubated with ABCA1 mutant (Q597R) cells remained in the monomeric form (Fig. 5, *lower panel*). Furthermore, we have reported that lipid-free apoE3 incubated with stimulated normal fibroblasts generated pre-β-LpE3 with a particle size ranging from 9 to 15 nm (28). Interestingly, we found that pre-β-LpE3 contains four and eight molecules of apoE3 per particle, whereas lipid-free apoE3 incubated with ABCA1 mutant (C1477R) remained in the monomeric form (data not shown). It is likely that the minimum functional unit of ABCA1 is a tetramer that lipidates four molecules of apoA-I at the same time, whereas the presence of eight molecules of apoA-I per particle could be explained by the close proximity of two homo-

tetrameric ABCA1 subunits. This is in agreement with our observation that a significant proportion of ABCA1 exists in living cells as an oligomer higher than tetramer (Fig. 1). Thus, the oligomeric ABCA1 complex generated multimeric nascent HDL particles regardless of the apolipoprotein acceptor.

We have assumed that the presence of LpA-I particles having either four or eight molecules of apoA-I per particle is an accurate reflection of the presence of different LpA-I subpopulations generated by the oligomeric ABCA1 complex. It is possible, however, that the heterogeneity of LpA-I particles was in part due to the cross-linking procedure itself (*i.e.* intermolecular cross-linking may have occurred between separate LpA-I particles, giving rise to apparent higher order oligomers of apoA-I that were not normally produced by ABCA1). Although this possibility cannot be totally excluded, experiments with different molar ratios of DSP to apoA-I and experiments involving a cross-linking at 37 °C or room temperature rather than at 4 °C did not result in significant alteration of the number of apoA-I molecules per particle.

Although ABCA1 mutants Q597R and C1477R were found to oligomerize normally (data not shown) and localized to the plasma membrane, they showed the total absence of binding to apoA-I (21, 23). These results indicate that the apoA-I lipidation defect observed in either Q597R or C1477R ABCA1 mutants is not caused by impaired oligomerization of ABCA1. Furthermore, C1477R, a naturally occurring mutant of ABCA1 in which cysteine 1477 within the second large extracellular loop is replaced with arginine (10), was found to dimerize normally. This suggests that cysteine 1477 is not essential for ABCA1 homodimerization. We are currently investigating the structural requirements for the ABCA1 transporter to form an oligomeric complex.

It is well documented that phosphorylation of a number of cellular receptors triggers their oligomerization, which modulates their function. Recent studies from our laboratory and others have shown that ABCA1 phosphorylation by the cAMP/cAMP-dependent protein kinase-dependent pathway plays an important role in the apoA-I lipidation process (21, 23, 32, 33). It is possible that apoA-I induces ABCA1 phosphorylation, allowing ABCA1 oligomerization. Although apoA-I binds to both the dimeric and the tetrameric ABCA1, the presence or absence of apoA-I molecules did not affect the oligomerization of ABCA1 in our cell culture model (Figs. 2*B* and 3*A*, respectively). More thorough investigations are required to establish definitively a possible role of apoA-I in the ABCA1 oligomerization process.

The molecular organization of apoA-I molecules within nascent LpA-I particles formed by the lipid translocase activity of the oligomeric ABCA1 complex has not yet been determined. However, because of the absence of cholesterol acyltransferase activity in the extracellular medium to convert unesterified cholesterol to cholesteryl ester, it is most likely that nascent LpA-I particles are discoidal. We have previously suggested that the α-electrophoretic mobility of LpA-I particles may be attributable to their high content in phosphatidylinositol (20, 34). However, it is possible that the high number of apoA-I molecules per particle as documented in the present study may contribute to increase the net negative charge of LpA-I particles and consequently cause their α-electrophoretic mobility. Although the spatial organization of apoA-I molecules within nascent α-LpA-I particles is unknown, Segrest *et al.* (35) published a computer model referred to as the "double belt" model for reconstituted LpA-I particles containing two molecules of apoA-I. In this model, two ring-shaped molecules of apoA-I are stacked on top of each other, forming a continuous amphipathic α helix that wraps around the perimeter of the phospholipid disc in an antiparallel orientation, resulting in the greatest potential for salt bridge connections between the two molecules. It is likely that the conformation(s) of two apoA-I molecules assumed on 96-Å discs might not be the same as that found on nascent LpA-I containing four or eight molecules of apoA-I. Of interest, the presence of heterogeneous subspecies of nascent α-LpA-I having both a different size and number of apoA-I molecules supports the idea that apoA-I conformations on discoidal particles are highly flexible (36). However, we wish to make clear that no attempt was made to use these models to give a definitive interpretation concerning the organization of apoA-I molecules within nascent α-LpA-I. Our model presented in Fig. 6 is a simple illustration of apoA-I lipidation by the oligomeric ABCA1 complex. The detailed structural organization of apoA-I within these nascent particles requires more thorough investigations, which are currently ongoing.

The results presented in this study provide a biochemical basis for a cellular apoA-I lipidation pathway that involves oligomeric ABCA1 complex in peripheral cells. This process plays *in vivo* a key functional role in the biogenesis of nascent HDL particles.

Acknowledgment—We thank Karl H. Weisgraber for kindly providing apoE3.

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